Inhibition of The Fracture Healing Process in Smokers:

Deregulation of Cellular and Molecular Milieu in Tibial Fracture Micro-Environment

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Thesis submitted to The University of Lincoln in part fulfilment for the degree of Doctor of Philosophy (Ph.D.)

December 2013

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ABSTRACT

Introduction: Tobacco smoking has been shown to have a detrimental impact on fracture healing and is often implicated in the non- and delayed-union of bone. Whilst numerous studies have concentrated on the demographic and clinical manifestations of fracture healing and smoking, very little analyses have been undertaken at the biochemical level. **Aims:** This research project will assess the impact of smoking on the cellular and molecular mechanisms of bone healing by analysing human tibial fracture haematomas using a variety of laboratory techniques. **Materials and methods:** *Cell culture:* Fracture haematomas (~2 mL) were collected from anaesthetised patients (*n*=48), who had sustained a fracture of the tibia. The semisolid material was explanted into 25 cm^2 non-coated tissue culture flasks and allowed to clot. Complete culture media was prepared and pipetted into the vessels, which were then placed in an incubator (37˚C; humidified 5% CO_2). After characterisation via immunocytochemistry (using antigen-specific markers to CD29, CD44, CD73, CD105, CD166 and CD34), isolated cells were counted using flow cytometry and proliferation rates were compared between non-smokers and smokers. Cigarette smoke extract (CSE) was manufactured based on the methods of Bernhard *et al.* (2004), in order to synthesise an *in vitro* smoking environment. Cells harvested from non-smokers (*n*=10) were cultured and infused with the CSE (2.5%) and proliferation and cell recovery rates were compared between the treated and untreated groups. Cellular nitric oxide levels and necrosis rates (using propidium iodide for the latter) were also compared in a similar way. An MTT assay was conducted to assess response to various concentrations of BMP-2 (10, 100 and 500 ng mL^{-1}) infusion in cell

cultures. *Molecular assays:* Serum was extracted from the haematomas and resolved on 16% acrylamide gels before staining with either Coomassie blue or silver nitrate. The resolved gels were photographed using a Kodak gel imaging system and unknown bands were identified using MALDI-TOF. Intracellular TGF-β and IL-6 were probed using antibodies, which were then analysed via flow cytometry in order to confirm the presence of these acute-phase proteins. In additional experiments, the ELISA was undertaken to quantify and compare the amounts of VEGF and IL-6 in haematoma serum and lysed fracture cells respectively. Biochemical testing on serum for b-ALP, albumin and total protein amounts in fracture serum was carried out, comparing smoker (n=10) and non-smoker (n=10) levels. **Results:** *Cell culture:* Spindle-shaped, fibroblast-like cells were visible in the primary culture, which were expanded through at least 10 further passages. The immunostaining of cells suggested a mesenchymal stem cell (MSC) lineage (CD29+, CD44+, CD73+, CD105+, CD166+, CD34-). Absolute cell counting using flow cytometry revealed marked proliferation of cells after 3 passages of culture. There was a reduction in the rate of proliferation of MSCs in smokers over 2 passages compared with non-smokers (-20%). A 5-day proliferation study in the CSE-treated vs. untreated cells showed a reduction in the rate of doubling in the treated group (-40% $p<0.05$). Cells showed a recovery response after CSE was withdrawn from culture, but was not significant. Cell necrosis analysis of CSE-treated vs. untreated cells showed that the CSE-treated cultures had a higher rate of necrosis than the untreated cells (CSE-treated 14.37% vs. untreated 7.98% $p<0.05$). Nitric oxide levels were lower in the CSE-treated cells (CSE-treated 3.0 μM vs. untreated 3.6 μM; $p<0.05$). MTT assay; BMP-2 infusions all improved cell viability compared to the non-infused cells,

with a BMP-2 concentration of 10 ng mL^{-1} increasing cell viability the most, though not significantly (+23%; $p>0.05$). *Molecular assay:* After staining, SDS-PAGE gels showed numerous bands of serum proteins and the haematoma serum lanes displayed unknown proteins which were later found to be haptoglobin (~20 kDa) and haemoglobin (~14 kDa) chains via mass spectrometry (MALDI-TOF). Cells were strongly positive (>96%) for the intracellular protein markers TGF-β and IL-6. The VEGF-A (serum) and IL-6 (cells) preliminary data from the ELISA revealed a reduction of these acute-phase proteins in patients who were smokers (VEGF-A -10%; IL-6 -15%). In the biochemical assays, albumin was reduced in smokers' serum (-13%, $p<0.05$), whereas b-ALP was raised (+20%; $p>0.05$ n.s.) and total protein lowered (-12%; $p<0.01$). **Conclusions:** The haematoma cultures produced colonies of adherent fibroblast-like cells that were of a MSC lineage. With the exception of a residual haemolysis-derived haptoglobin band, SDS-PAGE did not appear to show an over-expression of acute phase proteins, although it was useful for the characterisation of the fracture milieu, in that it was suggestive of a consistently haemolysed haematoma. The effect of smoking on bone fracture healing, therefore, appears to contribute to the inhibition of MSC proliferation, angiogenesis and the acute phase stress response. Cigarette smoke was also shown to cause excessive necrosis and reduce the amount of NO in those MSC cultures treated with CSE, which may indicate reduced vasodilatation to the fracture site. Bone alkaline phosphatase (b-ALP) was raised in the fracture serum of smokers, suggesting abnormally-high bone turnover, whilst the reduction of total protein and serum albumin likely indicates a lowered capacity for acute-phase protein synthesis and calcium transport in these patients. Exogenous BMP may be indicated for rectification of malunion in smokers.

ACKNOWLEDGEMENTS

I would firstly like to express sincere gratitude to my research project supervisory team, **Dr. Mohamed El-Sheemy[ǂ], Professor Oleg Eremin[ǂ]**, **Professor Mohammad Maqsood[*]** and **Dr. Carol Rea[#]** at the [ǂ]Research and Development Department, [*]The Department of Orthopaedic Surgery, Lincoln County Hospital and [#]The School of Life Sciences, The University of Lincoln for their invaluable and learned support, guidance and expertise during this research project.

I am truly grateful to **Dr. Issam Hussain**, **Mr. Neil Jelly and Mr. Khalid Arif** (University of Lincoln) for their esteemed help, professionalism and assistance with the experimental and technical aspects. Issam, Neil and Khalid have proved themselves to be excellent friends and extremely resourceful scientists; they were always on-hand to advise me about my ideas concerning the experimental design issues.

I would like to extend many thanks to the excellent scientific personnel, **Dr. Julian Bartrup, Mrs. Angela Murtagh**, **Mrs. Beverley Shepherd** (University of Lincoln), **Mrs Val Elliot** (Lincoln County Hospital) **Mr. Kevin Bailey** (University of Nottingham), **Dr. Chuthapisith Suebwong** (University of Nottingham) and **Dr. Mark Higgins** (Analytix, Durham), not forgetting **Mr. John Flynn** and **Mrs. Laura Pearson** for help and guidance in respect of the statistical and data analysis aspects.

In addition, I am indebted to the **Lincoln County Hospital Research and Development Committee** for generously funding this studentship, without whom, this study would not have been possible. **The Rotary Club of Darwen, Lancashire**, have also been very generous in their support by providing finance for my attendances at the European Wound Management Society's (EWMA) conference that was held in Helsinki in May 2009, the 5th Meeting of The European Tissue Repair Society, Limoges in August 2009, the European Society for Surgical Research (ESSR), 45th Annual Congress, Geneva, June, 2010 and the International Society of Orthopaedic Surgery and Traumatology; XXV Triennial World Congress in September 2011, Prague. I am extremely grateful to them also.

I take this opportunity to thank my viva voce team, **Professor Ted Fuller** (chair), **Dr. Sarah Cartmell** (external examiner, University of Manchester) and **Dr. Csanád Bachrati**[#] (internal examiner) for an extremely professional and comprehensive PhD examination. I was mightily impressed at the way proceedings were refereed by Professor Fuller and also by the depth of scientific knowledge of my examiners.

Thanks especially to my wife and son and my family and friends, for their continued patience, support and understanding throughout my PhD research studies.

Andrew Sloan

DECLARATION

The experimental procedures described in this report were carried out by the author, with some assistance from **Dr. Issam Hussain**, who provided expert hands-on support in the culture cabinet to minimise biohazardous contamination. The mass spectrometry (MALDI-TOF and lc-msms) assays, including gel tryptic digestion preparation, were undertaken by **Mr. Kevin Bailey** of the Biopolymer Synthesis and Analysis Unit (BSAU) at the University of Nottingham. Nitric oxide assays were performed by **Dr. Mark Higgins** of Analytix, Durham, with assistance from myself.

Andrew Sloan

RESEARCH PUBLICATIONS

CONFERENCE ABSTRACTS (Presenting author underlined)

Inhibition of Fracture Healing in Smokers: Cellular and Molecular Aspects. **Sloan A**, Hussain I, Sharma V, Maqsood M, Eremin O and El-Sheemy M. Society of Academic and Research Surgery (SARS) Annual Conference 2009 Bristol 7 - 8 January 2009 (Oral Presentation).

Inhibition of Fracture Healing in Smokers: Cellular and Molecular Aspects. **Sloan A**, Hussain I, Sharma V, Maqsood M, Eremin O and El-Sheemy M. The 19th Conference of the European Wound Management Association (EWMA) in co-operation with the Finnish Wound Care Society FWCS, Helsinki (Finland) 20 – 22 May 2009 (Poster Presentation).

Novel Approach of Effective Isolation of Mesenchymal Stem Cells from Human Umbilical Cord Blood. I. Hussain, A. Sloan, S. Magd, O. Eremin and M. El-Sheemy. European Society for Surgical Research (ESSR); 44th Congress. Nimes, (France) 19 – 24 May 2009 (Oral presentation).

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Novel Approach of Effective Isolation of Mesenchymal Stem Cells from Human Umbilical Cord Blood. I. Hussain, **A. Sloan**, S. Magd, O. Eremin and M. El-Sheemy. 5th Meeting of The European Tissue Repair Society (ETRS), Limoges 25 – 29 August 2009 (Oral presentation).

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The Effects Of Tobacco Smoking On Fracture Healing: A Model That Demonstrates Cellular Damage. **Sloan A,** Hussain I, Maqsood M, Eremin O and El-Sheemy M.; European Society for Surgical Research (ESSR), 45th Annual Congress, 9-12 June 2010 Geneva (Switzerland) (Oral presentation).

The Effects Of Tobacco Smoke On Fracture Healing: An *In Vitro* Model. **Sloan A,** Hussain I, Maqsood M, Eremin O and El-Sheemy M. International Society of Orthopaedic Surgery and Traumatology; XXV Triennial World Congress, 6-9th September 2011, Prague, (Czech Republic) (Oral presentation).

BOOK ENTRIES

Novel Approach of Effective Isolation of Mesenchymal Stem Cells from Human Umbilical Cord Blood. I. Hussain, A. Sloan, S. Magd, O. Eremin and M. El-Sheemy (2009) European Society for Surgical Research (ESSR) Proceedings, Nimes (France) ISBN 978-88-7587-506-0

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ABBREVIATIONS

Alb	Albumin
ACh	Acetylcholine
ACTH	Adrenocorticotropic Hormone
ALCAM	Activated Leukocyte Cell Adhesion Molecule
ATP	Adenosine Tri-phosphate
BAMBI	Bone Morphogenetic Protein and activin membrane bound inhibitor
BMP	Bone Morphogenetic Protein
b-ALP	Bone-Alkaline Phosphatase
CD	Cluster of Differentiation
Chd	Chordin
CSE	Cigarette Smoke Extract
CSF	Colony Stimulating Factor
CO	Carbon Monoxide
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetra-acetic Acid
ELISA	Enzyme Linked Immuno-sorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
FCS	Foetal Calf Serum
FGF	Fibroblast Growth Factor
FITC	Fluorescin Isothiocyanate
FOP	Fibrodysplasia Ossificans Progressiva
FST	Follistatin

FW	Formula Weight
G-CSF	Granulocyte-Colony Stimulating Factor
GDF	Growth Differentiation Factor
GREM	Gremlin
HCs	Haematoma Cells
HCN	Hydrogen Cyanide
HRP	Horse Radish Peroxidase
IFN	Interferon
IGF	Insulin-Like Growth Factor
IMDM	Iscove's Modified Dulbecco's Media
IL	Interleukin
ILGFBP	Insulin-Like Growth Factor Binding Proteins
ISCT	International Society for Cellular Therapy
LC-MSMS	Liquid Chromatography Tandem Mass Spectrometry
L-Glu	L-Glutamine
LMD	Listmode File
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionisation – Time-Of-Flight
MMP	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cell
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	Nitric Oxide
NOG	Noggin
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor

PLA_2	Phospholipase A_2
QTOF	Quaternary Time-of-Flight
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
Smurf	Smad Ubiquitin Regulatory Factor
SOST	Sclerostin
TGF-β	Transforming Growth Factor-Beta
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

Tobacco smoking was first shown to have a deleterious effect on tissue healing in 1977 by Mosely and Finseth, who reported an impaired digital blood-flow in the hand wound of a smoker. Indeed, the association of smoking and delayed wound healing, particularly in the field of orthopaedics, is now a well recognised caveat in clinical practice (Adams *et al.*, 2001; Patel *et al.*, 2013). Following the work of Mosley and Finseth, similar observations have been made in various other tissues including skin, bone, mouth and peptic ulcers (Silverstein, 1992; McRobert, 2013; Takamiya *et al.*, 2013). Despite this interest, the specific biochemical relationship of smoking and wound healing has not yet been fully elucidated (Whiteford, 2003). This is most likely the result of hospitalisation and immobility, which usually prevent continuation of smoking during the early recovery period following the trauma, thereby disallowing substantial viable human tissue procurement (Silverstein, 1992). One study reported that elective surgery patients were more likely to quit smoking if the advice came from their general practitioner rather than if the advice came from an anaesthetist, nurse or hospital literature (Webb *et al.*, 2013). The cost to the NHS of treating smoking-related disease is estimated to be approximately £5.2 billion per year (Callum *et al.*, 2010).

There are >4000 reported toxins in tobacco cigarette smoke, yet most research addressing the effects of smoking on tissue healing has emphasised the activity of the addictive component, nicotine (Siana *et al.*, 1989; Al-Hadithy *et al.*, 2012; Takamiya *et al.*, 2013). The pharmacokinetics of nicotine are considered to be a

causative factor in healing impairment due to both a capacity to prevent adequate vascular function, a consequence of nicotine's vasoconstrictive properties and an inhibitory action on macrophage and fibroblast proliferation (Silverstein, 1992; Tomek *et al.*, 1994; Tipton and Dabbous, 1995; Wong and Martins-Green, 2004). The process of tissue formation and development is essential in fracture healing and, therefore, requires adequate mesenchymal stem cell (MSC) production, as well as the migration and deposition of acute-phase proteins into the extracellular matrix (ECM) (Hynes, 1986; Dimitriou and Giannoudis, 2013). Thus, high plasma levels of nicotine and tobacco-associated toxins are likely to disrupt the bone healing process, because the rate of mesenchymal stem cell proliferation is reduced (Villarreal *et al.*, 1999; Patel *et al.*, 2013).

The process of fracture healing involves a cascade of complex, integrated, inflammatory and biomolecular processes that are initiated in response to injury. Bone tissue is repaired, regenerated and reformed during the classical three-phase, overlapping series of key biological processes termed *inflammatory phase*, *reparative phase* and *maturation phase,* or *bone remodelling*. The repair pathway consists of embryonic developments, with precisely co-ordinated participation from several cell types (Ferrara and Davis-Smyth, 1997; Dimitriou *et al.*, 2005). Importantly, there are specific adhesive interactions between cells and the extracellular matrix (ECM) in their immediate microenvironment (Ettinger and Doljanski, 1992; Raghow, 1994; Bainbridge, 2013).

There are many similarities shared between fracture healing and embryogenesis. Wound healing following an acute injury such as fracture, regardless of the

mechanism, recapitulates the events associated with the development of the zygote *in utero* and there is a heightened biosynthetic activity at the site of repair (Tranquillo and Murray, 1992; Dimitriou *et al.*, 2011). Aside from their role as crucial mechanisms in healing, cell adhesion and migration are integral factors in organogenesis during embryonic and neural development, tumour metastases and post-inflammatory tissue repair (Howell, 1992; McGowan, 1992; Yost, 1992; Le Bras *et al.*, 2012).

This introduction will briefly focus upon the basic anatomy and physiology of bone and fracture, including the hormonal aspects, before analysing the cellular and molecular features of fracture healing and how tobacco smoking elicits such a detrimental impact on the events that are associated with the repair processes. It will describe the cytokine involvement that mediates the regeneration of injured limbs and how they adeptly switch on and switch off vital cellular actions. The information available in the literature concerning the effects of smoking has been sourced to convey the main issues associated with malunion that is hampering patients' recovery after sustaining a fractured limb.

1.1 OVERVIEW OF OSTEOLOGY

1.1.1 Bone Physiology

Bone is made up of several different tissue types comprising of osseous tissue, cartilage, dense connective tissue, epithelium, adipose tissue and nervous tissue (as summarised in Table 1). Each bone in the skeleton is classed as an individual organ on account that all these different tissue types work together, each being dynamic and highly complex. The process of bone remodelling, or turnover, is continual throughout life and concerns all the different types of bone cells (Clarke, 2008).

Table 1: Function of Bone

Function	Description
Support:	Supports the tissues of the body by providing a structural framework on which muscle, tendons and ligaments are attached.
Protection:	Many internal organs are protected from injury by bone. Ribs protect visceral organs such as the heart, lungs, spleen and most of the liver; the cranium houses the brain.
Assistance in movement:	Bones are vital in movement since they are attached to muscles.
Mineral homeostasis:	Calcium (Ca^{+2}) and phosphorous (P) are stored in bone and released into the blood on demand to maintain critical mineral balances. Ca^{+2} and P form hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals; also present in the amorphous form.
Blood cell production:	Haematopoiesis takes place in the red bone marrow; mainly pelvis, ribs, breastbone, vertebrae, femur and skull. Red bone marrow consists of reticulocytes, adipocytes, fibroblasts and macrophages within a network of reticular fibres.
Triglyceride storage:	Triglycerides are stored in yellow bone marrow as a chemical energy reserve. Adipose cells store triglycerides and blood cells.

(Tortora and Grabowski, 2003)

Table 2: Structure of Bone

Structure	Description
The diaphysis:	The long cylindrical shaft that forms the main portion of the bone.
The epiphyses:	The distal and proximal ends of the bone.
The metaphyses:	The regions where the diaphysis joins the epiphyses and includes an epiphysial plate in growing bones. This allows for growth in length and is replaced by a structure known as the epiphysial line on cessation of growth.
Articular cartilage:	A thin layer of hyaline cartilage covering the epiphyses where the bone forms an articulation point with another bone. Its purpose is to reduce friction and absorb shock at freely moveable joints. Repair of the cartilage is restricted because there is no perichondrium.
The periosteum:	A hard sheet of dense irregular connective tissue that covers the bone surface where it is not covered by articular cartilage. This type of tissue contains the bone forming cells that enable the bone to grow in diameter and thickness, but not in length. The periosteum assists in fracture repair, nourishment of bone and serves as an attachment point for ligaments and tendons.
The medullary cavity:	The space within the diaphysis that houses the yellow bone marrow that contains the triglyceride fats storage.
The endosteum:	The thin membrane lining the medullary cavity that contains osteoclasts and a small amount of connective tissue.

(Tortora and Grabowski, 2003)

Figure 1: Anatomy of a Long Bone

The following diagram (Figure 1) is a schematic representation of the femur, displaying epiphyses, diaphysis, articular cartilage, medullary cavity, periosteum and cancellous and compact bone:-

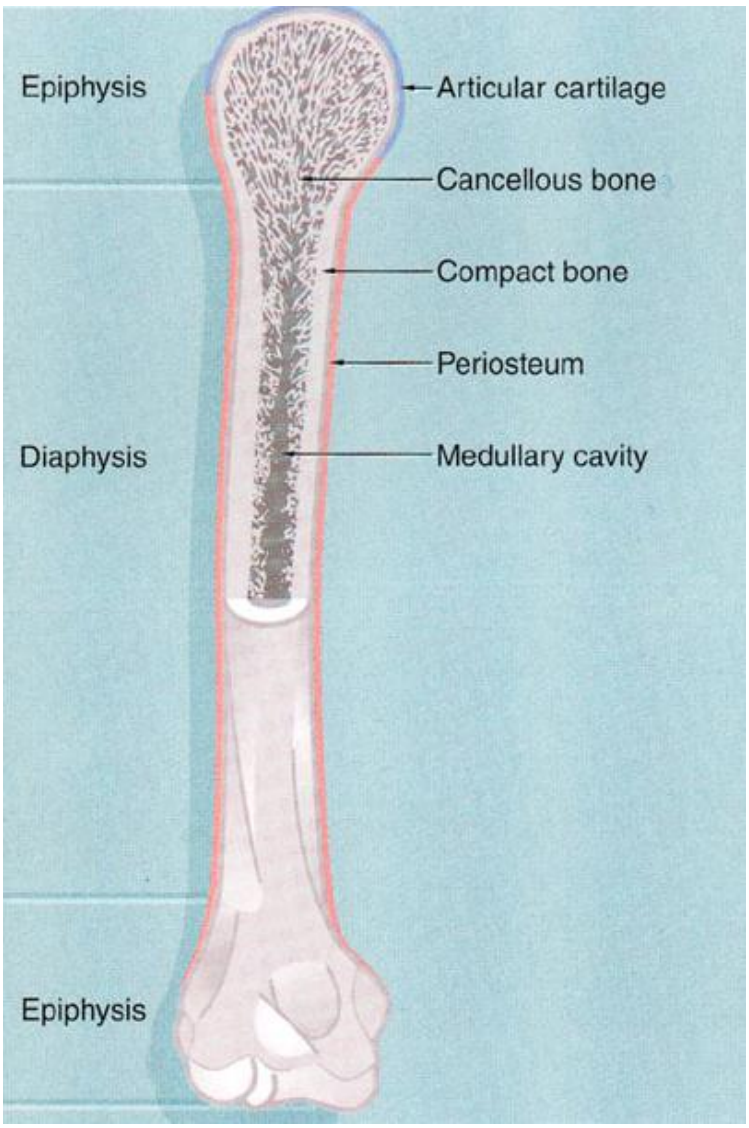

Figure 1: Diagram of a long bone
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(Young and Heath, 2003, Pg. 176)

1.1.2 Histology of Bone

Types of histological bone tissue can be classified in several ways and is based on texture. The following diagram (Figure 2) illustrates a section of compact bone and the various systems in place.

Figure 2: Cross Section of Compact Bone

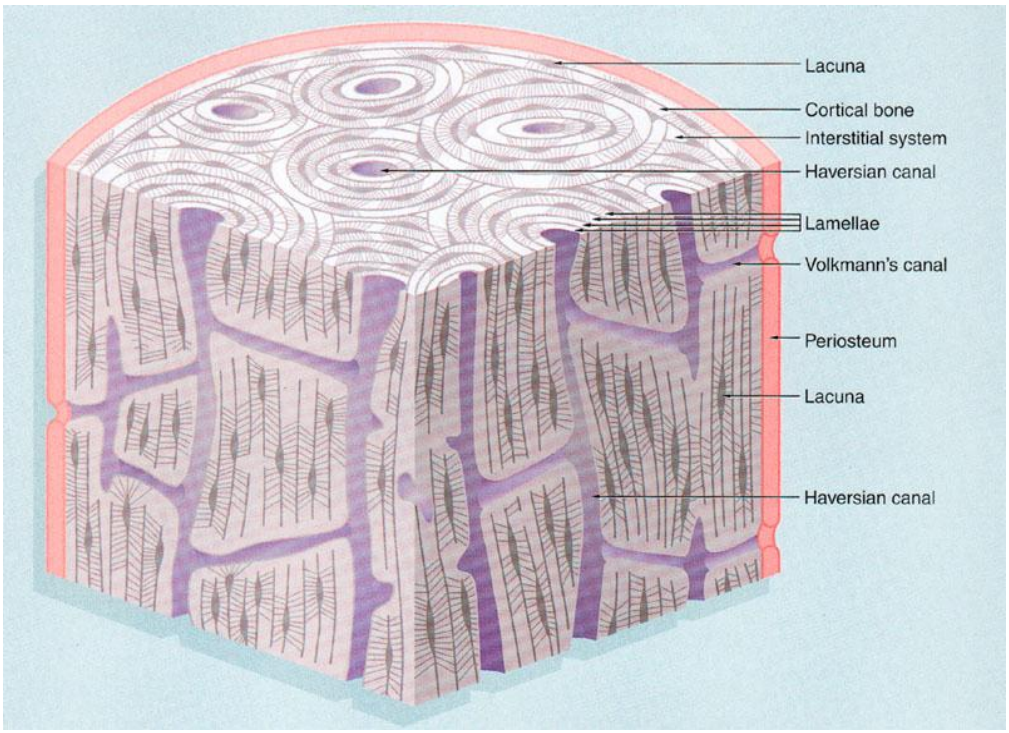

Figure 2: Cross section of compact bone

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(Young and Heath, 2003, Pg.177)

Table 3: Histology of Bone

Histological feature	Description
Compact bone:	Ivory-like bone, dense in texture and without cavities. Forms the shell of many bones and surrounds the trabecular bone in the centre. Consists mainly of Haversian systems or secondary osteons, which are neurovascular channels interconnected via the obliquely piercing Volkmann's canals. The parallel bony columns are arranged along the lines of stress exerted on the bone.
Spongy bone:	Spongy bone has numerous cavities and is located within the medullary cavity. It consists of extensively connected bony trabeculae oriented along the lines of stress. Includes trabecular bone, cancellous bone. In contrast to compact bone, complete osteons are normally absent due to the thinness of the trabeculae. Sponge bone is more metabolically active than compact bone due to its much larger surface area for remodelling.
Lamellar bone or secondary bone tissue:	Lamellar bone is mature bone with collagen fibres arranged in lamellae. In spongy bone, lamellae are arranged parallel to each other, whereas in compact bone, they are concentrically organised around a vascular canal, termed a Haversian canal.
Woven bone or primary bone tissue:	Woven bone is immature bone with collagen fibres arranged in irregular random arrays and contains smaller amounts of minerals but a higher proportion of osteocytes than lamellar bone. Woven bone is temporary and is eventually converted to lamellar bone. It is pathologic tissue in adults except in a few places such as the areas near sutures of the flat bones of the skull, tooth sockets and the insertion sites of some tendons.
Immature bone or primary bone tissue:	Immature bone is woven bone.
Mature bone or secondary bone tissue:	Mature bone is lamellar bone and almost all bones in adults are lamellar bones.
Intramembranous bone or mesenchymal bone:	Intramembranous bone develops from a direct transformation of condensed mesenchyme and flat bones are formed in this way.
Intracartilaginous bone, cartilage bone or endochondral bone:	Intracartilaginous bone is shaped by replacing a reformed cartilage model. Long bones grow in a longitudinal direction, acting on the epiphyseal growth plate.

(Kobayashi *et al.*, 2003; Clarke, 2008)

1.1.3 Bone Cytology: Osteoblasts

Osteoblasts are located on the surface of bone (osteoid) and are responsible for synthesising the organic components of bone matrix (see Figure 3), including type I collagen, proteoglycans and glycoproteins. Osteoblasts also synthesise the enzyme alkaline phosphatase, which is needed locally for the mineralisation of osteoid (Rivera *et al.*, 2013). Active osteoblasts are of cuboidal or columnar shape with eccentrically located nuclei and a perinuclear cytoplasmic halo. Inactive osteoblasts are flattened with low alkaline phosphatase activity. Osteoblasts contact their neighbouring osteoblasts cytoplasmically and do not divide but rather mature into osteocytes. They remain as osteoblasts or return to the state of osteoprogenitor cells from which they are derived (Kobayashi *et al.*, 2003; Clarke, 2008).

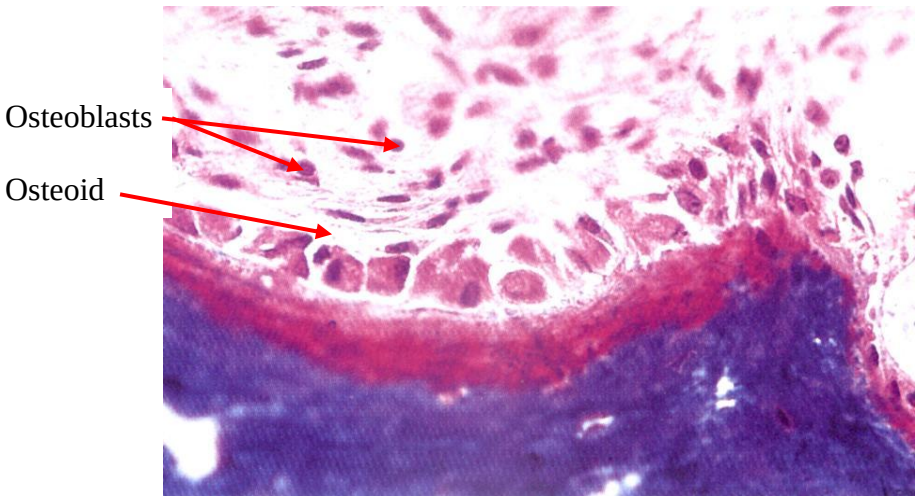

Figure 3: Osteoblast and Osteoid in a Section of Bone

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(Young and Heath, 2003, Pg.181)

1.1.4 Bone Cytology: Osteocytes

Osteoblasts undergo maturity into osteocytes when encased by the self-synthesised osteoid matrix. Lacuna and canaliculi form around osteocytes and their cytoplasmic processes. Osteocytes lie in their own lacuna and contact neighbouring osteocytes cytoplasmically through the canaliculi. Adjacent cells make contact via gap junctions, maintaining the strength of osteocytes by passing nutrients and metabolites between blood vessels and distant osteocytes. This process regulates ion homeostasis and transmits electrical signals in bone. Although osteocytes have a reduced synthetic activity and are not capable of mitotic division, they are actively involved with the maintenance of bony matrix. Some of the osteocytes die during remodelling but most return to the state of osteoprogenitor cell or persist as osteocytes in the long term (Clarke, 2008).

Figure 4: Osteocytes in Cancellous Bone

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(Young and Heath, 2003, Pg.182)

1.1.5 Bone Cytology: Osteoclasts

Osteoclasts are derived from a monocytic-macrophage system and are responsible for bone resorption. They are multinucleated cells with fine finger-like cytoplasmic processes and are rich in lysosomes containing tartrate-resistant acid phosphatase (TRAP). Osteoclasts lie in resorption craters known as Howship lacunae on bone surfaces or in deep resorption cavities known as cutting cones. They can only resorb mineralised bone matrix (Reddy, 2004).

Osteoclast activity always predominates over that of osteoblasts because they are three times more efficient at bone resorption than osteoblasts are at bone deposition. On balance, osteoclasts have a much shorter life span than osteoblasts. Osteoclasts are rarely seen in routine histological sections of normal bone. An increased number of osteoclasts are characteristic of disease associated with increased bone turnover, such as osteoporosis. Osteoclasts do not have receptors for parathyroid hormone but do have receptors for calcitonin and the hormonal signal is mediated by osteoblasts (see later) (Martin and Sims, 2005).

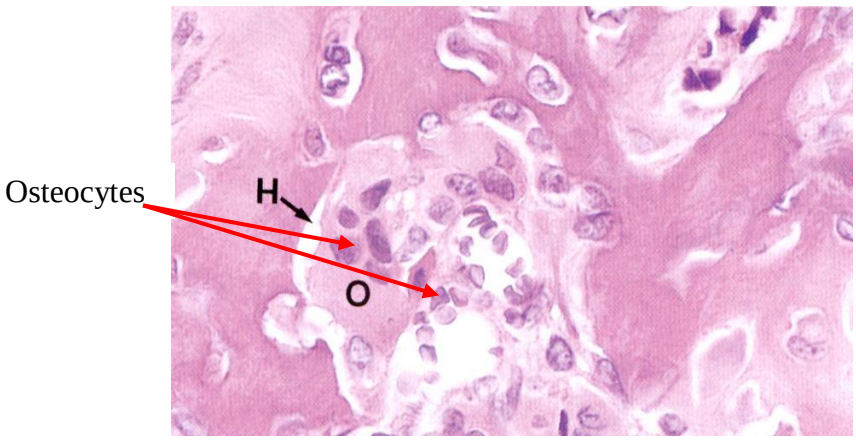

Figure 5: Osteoclasts

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(Young and Heath, 2003, Pg.181)

1.2 OVERVIEW OF FRACTURE HEALING

A fracture is simply defined as any break in a bone. Despite a generous blood supply, fractures can take a considerable amount of time to heal and one study reported an average time to union of six months (Marcacci *et al.*, 2007). This is due to the fact that calcium and phosphorous, necessary to strengthen and harden new bone, are deposited very gradually during the process. In severely fractured bones, the temporary disruption in the blood supply accounts for elongated healing times (Dimitriou *et al.*, 2005).

Fracture healing comprises a complex series of sequential interrelated biomolecular processes that involve both intracellular and extracellular signalling processes during the repair and regeneration of damaged bone. This procedure follows a chain of events that are specifically orientated to temporal and spatial sequences (Sandberg *et al.*, 1993; Reddi, 2001; Lee *et al.*, 2004; Marsell and Einhorn, 2011; Dimitriou and Giannoudis, 2013). The molecular mechanisms responsible for mediating embryonic development are replicated in skeletal tissue formation during fracture healing. Bone, therefore, has the unique ability to regenerate without the permanent formation of a scar, a process that is seen in the repair of tissues in the developing mammalian foetus and is achieved via complex overlapping phases of healing. Indeed, fibrous scars compromise the mechanical integrity and strength of the skeleton. A healed bone will often be stronger than it was before the fracture; a phenomenon known as 'Wolff's Law', which is named after the German surgeon Julius Wolff (1836-1902). Wolff observed that long

bones change shape to accommodate stresses placed on them (Prendergast and Huiskes, 1995; Kanczler and Oreffo, 2008; Wolff, 2010).

Despite some reported variations on the theme, there exists a multi-phased fracture repair pattern, or classical 'three-phase dogma', consisting of an *inflammatory phase*, a *reparative phase* and *maturation*, or *remodelling phase*, which have been historically well characterised in the literature (Schilling, 1976; Howell, 1992; Marcacci *et al.*, 2007). There is a shared heuristic regulatory similarity that includes the extra-cellular matrix (ECM), cytokines, receptors, signalling molecules and transcription factors (Yu *et al.*, 2002; Takayanagi, 2005).

There are four separate tissue components involved in long bone fractures; the cortex, the periosteum, the bone marrow and the external soft tissues. The extent of involvement of each component is dependent upon the following factors present at the site of injury;

- Level of growth factors
- Hormones
- Nutrients
- pH of the microenvironment
- Oxygen tension of the microenvironment
- Electrical environment
- Mechanical stability of the fracture itself

(Riedel and Valentin-Opran, 1999)

1.2.1 Types of Fracture

There are several types of fracture, named depending on severity, the position or after the surgeon who originally described them. The most common long-bone fractures are as follows:

- **Open or compound fracture:** The fractured portion of the bone protrudes through the skin.
- **Comminuted fracture** (Figure 6): The bone splinters and fragments at the location of impact and pieces of bone lie *in situ* of the fracture.
- **Greenstick fracture:** A partial fracture that occurs in children, where one side of the bone is broken and the contralateral side is bent.
- **Spiral Fracture** (Figure 7): Sustained due to a twisting force on the bone.
- **Impacted fracture** (Figure 8): One end of the fracture is driven into the interior of another
- **Pott's fracture** (Figure 9): A fracture of the distal and lateral portion of the lower leg, resulting in serious injury of the distal tibial articulation.
- **Colle's fracture**: A fracture of the distal radius bone of the forearm, in which the distal fragment is displaced posteriorly.

(Tortora and Grabowski, 2003)

1.2.2 Types of Fracture in the Study

The following radiographs, sourced from participants, illustrate the most common types of tibial fracture presented by patients involved in this study:

Figure 6: Comminuted Fracture

Figure 7: Spiral Fracture

Figure 8: Impacted fracture

Figure 9: Pott's Fracture

1.2.3 The Extracellular Matrix

The ECM has an intimate involvement with all aspects of bone repair and remodelling, especially in callus development and post-inflammatory regeneration. The dynamically-modulated key stages involved in healing are accomplished through a unique capability of the ECM to generate vast arrays of structures, offering support in an injured environment. The matrix has the capacity to orchestrate cellular processes, bind specific growth factors and cytokines and organise the geometric framework that can assist in cell migration as well as numerous cell-to-cell interactions (Raghow, 1994; Pankov and Yamada, 2002; Hidalgo-Bastida and Cartmell, 2010).

The specialised adhesive cell-to-cell interactions are enabled by the substantial range and abilities of cell surface receptors and integrins; the latter being generated by various combinatorial associations amongst the constituent α and β integrin chains, the receptors that mediate the attachment of a cell to the surrounding tissues around it (normally other cells and the ECM scaffold itself). The dynamic cell-to-ECM interactions are continuously modified by a range of potent cytokines and are subject to feedback regulation mediated by transcriptional, post-transcriptional, translational and post-translational mechanisms elaborated during fracture repair. The mediators involved in cell-to-ECM relationships include mesenchymal stem cells (MSCs), fibroblasts, endothelial and epithelial cells, as well as cells of the immune system (Raghow, 1994; Pankov and Yamada, 2002).

Figure 10: Biochemical Cell-to-ECM Interactions

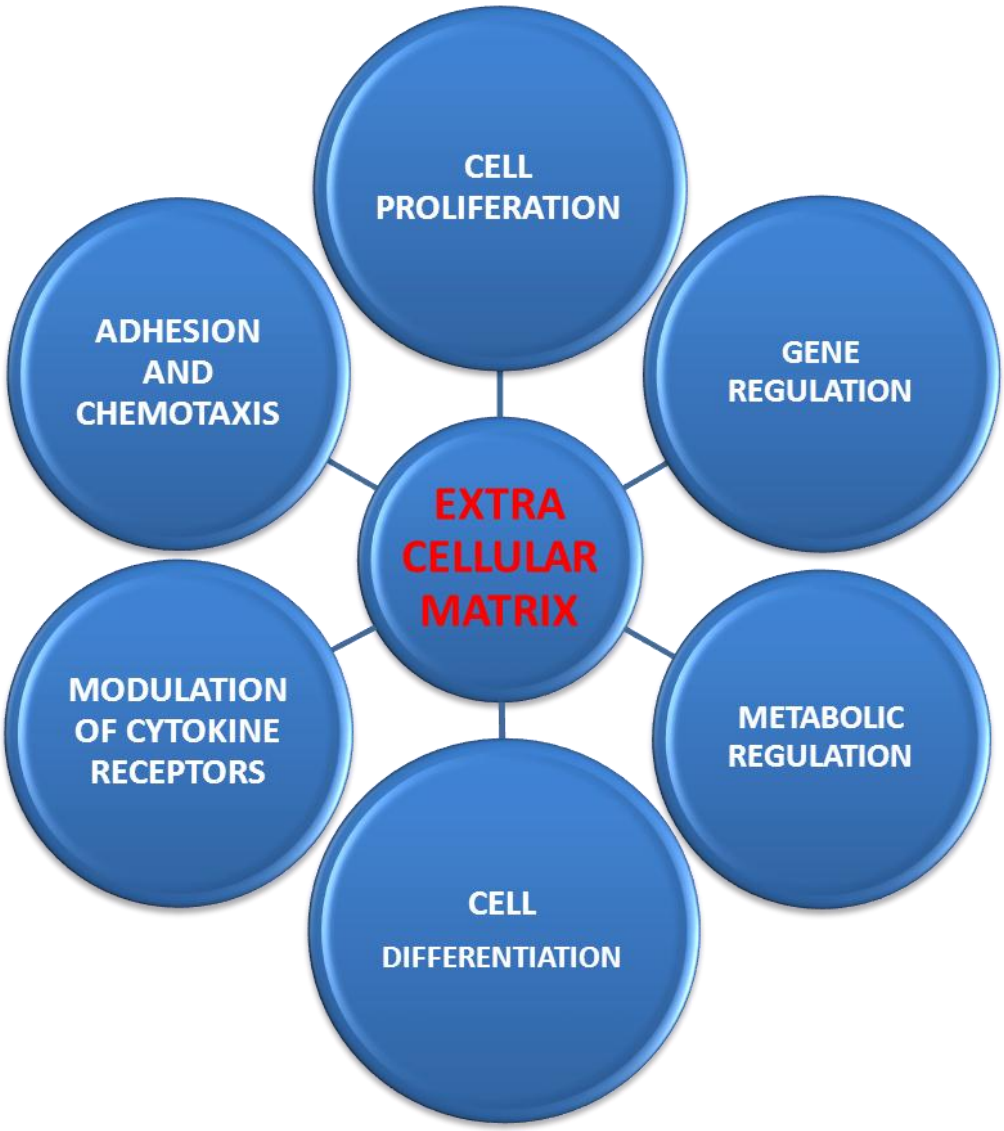

Figure 10. The cell-extracellular matrix relationship elicits many key mechanisms involved in post-inflammatory fracture healing and bone modelling. The ECM is a source of a variety of mediators, which impact on cellular processes in a dynamic reciprocity. Examples of these mediators include collagens, fibronectin, laminin, fibrinogen, vitronectin, thrombospondin, tenascin and proteoglycans. Cells often alter the quality and quantity of various ECM components they synthesise and are subsequently expressed into the interstitial fluid within the fracture micro-environment (Pankov and Yamada, 2002; Hidalgo-Bastida and Cartmell, 2010).

1.2.4 Regulatory Hormones in Fracture Healing

Bone is the body's largest reservoir of calcium (Ca^{2+}), with 99% being stored in the skeleton. Calcium blood levels are regulated by controlling resorption from bone to blood and its deposition from blood into bone. Many homeostatic processes, as well as haemostasis and enzymatic reactions, depend on stable Ca^{2+} levels within extracellular fluid, which are typically 9-11 μg mL^{-1} (2.2-2.5 mmol L^{-1}) in blood plasma (Tortora and Grabowski, 2003). Bone therefore acts as a buffer in calcium homeostasis, a process that is regulated by hormones (Fleet and Schoch, 2010).

1.2.4.1 Parathyroid Hormone, Calcitriol and Calcitonin

The most important hormone in Ca^{2+} regulation is parathyroid hormone (PTH), which is secreted by the parathyroid glands. Secretion of PTH operates via a negative feedback system, i.e. if a stimulus should trigger Ca^{2+} levels to fall, as is the case in early fracture, parathyroid gland cell receptors detect this change and increase their production of cyclic adenosine monophosphate (cAMP). The increased production of cAMP causes an increase in PTH synthesis, via the gene for this hormone in the nucleus of the parathyroid gland, releasing more PTH into the blood. The occurrence of higher levels of the circulating hormone stimulate osteoclastic activity, speeding up the rate of bone resorption which gives rise to elevated levels of Ca^{2+} in the blood. Hence, blood calcium levels return to normal due to its release from the bone (Jilka, 2007; Paskalev and Goranov, 2012; Aspenberg, 2013).

Calcium levels may not necessarily be low following fracture in certain patients. Serum parathyroid hormone and calcium levels were measured in elderly hip fracture patients (*n*=20) in a study conducted by Sato *et al*. (2001). At one week following injury, parathyroid hormone was found to be depressed, whilst Ca^{2+} levels were increased. It was shown that both these analytes returned to normal after three months post-injury. The group found that increased bone resorption and decreased bone formation with hypercalcaemia are present by one week following the hip fracture and some heightened resorption continues for at least 3 months. This quandary could explain why elderly patients are of high risk of sustaining another hip fracture (Sato *et al.*, 2001).

Parathyroid hormone also acts upon the kidneys to slow down the rate of Ca^{2+} loss in the urine, in order that more is retained in the blood. In addition, it promotes synthesis of calcitriol, a hormone that stimulates absorption of calcium from the gastrointestinal tract, thus assisting in elevating Ca^{2+} levels further. The hormone calcitonin (CT) also contributes to the homeostasis of Ca^{2+} in the blood in that it has the opposing effect of PTH. In the event that blood calcium levels become raised, parafollicular cells of the thyroid gland secrete CT, which inhibits osteoclastic activity, thereby accelerating Ca^{2+} deposition into bone. Calcitonin consequently promotes bone formation and reduces blood Ca^{2+} levels (Tortora and Grabowski, 2003; Fleet and Schoch, 2010).

1.2.4.2 Oestrogen and Osteoporosis

Osteoporosis is a bone disease that is epitomised by increased porosity of the skeleton due to a reduction in bone mass. The associated structural changes in bone architecture give rise to a heightened risk of fracture. The condition may be localised to a single bone or region, as in *disuse osteoporosis of a limb*, or may involve the entire skeleton, as a manifestation of a metabolic bone disease (McNamara, 2010).

In the ten years following menopause, which is characterised by a reduction in the production of oestrogen, yearly reductions in bone mass can reach 2% of cortical bone and 9% of cancellous bone. Within the 30-40 years following menopause, women can lose up to 35% of cortical bone and 50% of their trabecular bone. It is for this reason that postmenopausal women are at an extremely high risk of sustaining an osteoporotic fracture than men. Postmenopausal osteoporosis can be described as a hormone-dependent acceleration of bone loss due to oestrogen deficiency. Indeed, oestrogen replacement at menopause is protective against such bone depletion. The effect of this hormone on bone mass is mediated by cytokines and decreased oestrogen levels give rise to increased secretion of IL-1, IL-6 and TNF-α by blood monocytes and cells of the bone marrow. Such cytokines are potent stimulators of osteoclast activity and recruitment. A degree of compensatory osteoblastic activity does occur, but fails to keep up with the osteoclasts, resulting to what can be described as a high turnover form of osteoporosis (Raisz, 2005; D'Amelio *et al.*, 2008; Faienza *et al.*, 2013).

1.2.4.3 Vitamin D (Cholecalciferol)

Vitamin D, also known as cholecalciferol, is synthesised by natural sunlight exposure to the skin and is responsible for the evolution of a healthy calcified skeleton; it is a hormone that elicits a wide range of biological processes. The ultraviolet B radiation (UVB) in sunlight converts 7-dehydrocholesterol to pre-vitamin D_3 which rapidly becomes isomerised to vitamin D_3. Once synthesised, it is metabolised in the liver to 25-hydroxyvitamin D_3 and in the kidneys to its active form 1,25-dihydroxyvitamin D_3. 1,25-dihydroxyvitamin D_3 interrelates with its vitamin D receptor in Ca^{2+}-mediating tissues to regulate calcium metabolism and bone health. It is known that the majority of cells in the body have a vitamin D receptor and are capable of producing 1,25-dihydroxyvitamin D_3. 1,25-dihydroxyvitamin D regulates a wide array of genes that have essential functions in modulating cellular development, immune processes and cardiovascular homeostasis. Vitamin D deficiency is associated with a heightened risk of chronic conditions including autoimmune and cardiovascular disease, cancer and type II diabetes amongst others. Deficiency and insufficiency have been characterised as a 25-hydroxyvitamin D level of <20 ng mL^{-1} and 21-29 ng mL^{-1} respectively. Adults require 2000 IU of vitamin D a day to meet the body's vitamin D requirement. It is estimated that 1 billion people are vitamin D deficient or insufficient worldwide (Holick, 2011).

Fu *et al*. (2009) studied the efficacy of exogenous 1,25-dihydroxy vitamin D_3 on osteoporotic fracture healing, employing an *in vivo* rat model to evaluate its histomorphometric effects on bone repair. Six month old oopherectomised female Sprague Dawley rats (n=40) were randomised into two groups and a bilateral

midshaft femoral osteotomy was performed on each of the animals. Treatment in the groups commenced at the second day after fracture and was continued until sacrifice at 6 and 16 weeks post-osteotomy. The experimental group (n=20) was given 1,25-dihydroxy vitamin D_3, 0.1 μg kg^{-1} day^{-1} and the control group (n=20) was given a vehicle of middle chain triglyceride (MCT), both by oral gavage. Fracture callus in the rats was evaluated via a range of imaging techniques (x-ray and micro-computed tomography (micro-CT)) as well as histological testing. Biomechanical testing was also performed. At 6 weeks post-fracture, x-ray revealed a less pronounced fracture line in the 1,25-dihydroxy vitamin D_3 group compared with the MCT-vehicle group. However, the fracture line was not visible in both groups after 16 weeks. Micro-CT showed that the total volume of callus was 23% higher in the experimental group than that in the control group ($p<0.001$) after 6 weeks. Histology showed improved remodelling of the fracture callus in the 1,25-dihydroxy vitamin D_3 compared to the control group. Biomechanical performance was also superior in the rats that were given 1,25-dihydroxy vitamin D_3 (data not shown). Fu and co-workers concluded that exogenous 1,25-dihydroxy vitamin D_3 could enhance fracture healing by improving the histomorphometric parameters and mechanical strength of the fracture callus, giving rise to increased transformation of woven bone into lamellar bone (Kellum *et al.*, 2009).

1.3 THE PHASES OF FRACTURE HEALING

There are essentially three phases of fracture repair, the inflammatory phase, the reparative phase and the maturation, or remodelling phase. An overview of these phases is discussed as follows.

1.3.1 Inflammatory Phase

Immediately following the fracture, a haematoma is formed at the site of injury and the inflammatory phase is initiated. The haematoma results from traumatic damage to the vasculature, associated with musculoskeletal damage, acute necrosis and hypoxia of the surrounding tissues (Glowacki, 1998). The activation of the thrombotic factors within the coagulation cascade leads to the development of the haematoma at the site of the fracture. The early trauma period is characterised by the presence of certain mediators within the haematoma microenvironment, which includes platelets, cytokines, fibroblasts and mesenchymal stem cells (MSCs) associated with growing capillaries (Marsell and Einhorn, 2011).

Platelets are mobilised by thrombin and subendothelial collagen, ultimately expressing PDGF and TGF-β, factors that are actively involved with the initiation of fracture repair (Connor and Evans, 1982). There is marked proliferation and differentiation of mesenchymal cells (of osteogenic and chondrogenic lineage) over the subsequent days, during which time angiogenesis takes place, which is essential for the progression to the next stage; the reparative phase (Yu *et al.*, 2002).

The preliminary inflammatory step in bone healing is blood clotting, or haemostasis, which follows an initial momentary period of vasodilatation and increased vascular permeability immediately after the injury has taken place. The inflammatory phase can last up to 4 days from the onset of the fracture. Haemostasis provides the foundation from which the proceeding elements of the cascade can follow. Bleeding is rapidly controlled by acute vasoconstriction of the damaged vessel, prompting the endothelium and neighbouring platelets to trigger the intrinsic coagulation pathway. During this process, a fibrin clot, known as a haematoma, is formed; this contains collagen, platelets, thrombin and fibronectin. This haematoma is able to release various cytokines and growth factors into the ECM to act as inflammatory promoters. The early phase in trauma is known as the *acute-phase response* or *acute-phase reaction*, which triggers a systemic feedback cascade, involving the release of *acute-phase proteins* by the liver (Witte and Barbul, 1997; Broughton *et al.*, 2006).

The haematoma is able to provide a network of intricate scaffolding, which can be utilised by migrating leukocytes such as neutrophils, monocytes and fibroblasts and also endothelial cells during the acute-phase response. The liver plays an important role by regulating the amount of recruited cytokines and growth factors at the site of injury, which, if up or down-regulated by at least 25% during the inflammatory process, are termed acute-phase proteins. Those which are under-produced are termed *negative* acute phase proteins (e.g., serum albumin); those which are overproduced, (e.g., IL-6), are known as *positive* acute-phase proteins. Any deficiencies in those factors that are required for clot-formation (e.g., factor

XIII) give rise to impaired wound healing (Kurkinen *et al.*, 1980; Hollenbeck, 1996; Wong and Martins-Green, 2004).

1.3.1.1 Chemotaxis

As soon as the haematoma is formed, cellular signalling will elicit a neutrophil response. In addition, inflammatory mediators accumulate, prostaglandins (PGs) are activated and localised vasodilatation occurs, which facilitates cellular migration to the site of injury. The neutrophils are mobilised by various cytokines, including IL-1, TNF-α and TGF-β. Bacteria-produced lipopolysaccharides (LPS) are also capable of evoking similar responses (Bevilacqua *et al.*, 1985; Pohlman *et al.*, 1986; Gerstenfeld *et al.*, 2003; Dimitriou *et al.*, 2011). In addition to neutrophils, monocytes are activated and migrate to the wounded area, where they are transformed into macrophages. This is a crucial process for the transition to the early reparative phase of wound healing and usually occurs around 48-96 hours post-injury. An activated macrophage will then initiate angiogenesis by synthesising VEGF, FGF and TNF-α. The formation of fibrous tissue, or fibroplasia, occurs as a result of TGF-β, endothelial growth factor (EGF), PDGF, IL-1 and TNF-α synthesis. Indeed, IL-1 and TNF-α are accountable for the synthesis of nitric oxide (NO) through the activation of inducible nitric oxide synthase (iNOS), thus affording the initiation of vasodilatation (Witte and Barbul, 2002; Marsell and Einhorn, 2011).

1.3.1.2 Neutrophils and Macrophages

Neutrophils migrating to the wound site clear the area of residual bacteria and cellular debris. The neutrophils release proteolytic enzymes and proteases that digest bacteria and other debris and are therefore vital components of the fracture healing process. Proteases are categorised according to their specific target, whether it is proteins, amino acids or metal ions within enzymes. Serine proteases, for example, have a broad specificity, whereas metalloproteinases (which contain zinc ions) have a limited specificity dissolving both collagen and the ECM. Proteases break down the matrix at the wound site, as protease inhibitors (normally found in undamaged tissue) are overwhelmed and compromised during the inflammatory process due to the substantial release of the proteases (Yager and Nwomeh, 1999; Tsiridis *et al.*, 2007).

Neutrophils are also capable of generating reactive oxygen free radicals via a myeloperoxidase pathway, which are combined with chlorine to form a very effective sterilising agent and this is able to control the pathogen levels within the wound milieu (Yager and Nwomeh, 1999). There is widespread neutrophil apoptosis, which are subsequently replaced by macrophages, although the stimulus for this enhanced cell death is unknown. The macrophages *phagocytose* the dead neutrophils, clearing them from the injury site. Macrophages continue to destroy residual bacteria because they are able to produce large quantities of NO, a process stimulated by TNF and IL-1. A higher yield of NO can be further generated by a reaction between TNF and IL-1 and peroxide ion oxygen radicals to produce very toxic peroxynitrite and hydroxyl radicals (Goldman, 2004). The impaired ECM is cleared of any remaining inflammatory debris by matrix

metalloproteinase (MMP), an enzyme that is expressed by keratinocytes, fibroblasts, monocytes and macrophages in the presence of TNF-α (Abraham *et al.*, 2000).

The inflammatory phase is a well organised and coordinated process, mediated by a series of reactions that eventually give rise to *stop signals*, which are also known as *checkpoint controllers of inflammation* (Nathan, 2002; Serhan and Chiang, 2004). The major mediators involved in the regulation of inflammation are the various eicosanoids (e.g. PGE_2, PGI_2, PGF_2, TxA_2). The enzyme lipoxygenase, involved in the synthesis of eicosanoids, has a pivotal role in the synthesis of lipoxins. Lipoxins and aspirin-triggered lipoxins, are the stop signal for inflammation and are formed by platelets and leukocytes through transcellular biosynthesis (Broughton *et al.*, 2006). When platelets and neutrophils adhere to one another, the latter produces leukotriene A_4 (via 5-lipoxygenase), which is conveyed to the platelet and transformed into lipoxin A_4 and B_4 (Serhan and Chiang, 2004) and these are discussed in more detail later.

1.3.2 Reparative Phase

The second stage of fracture healing, occurring from day 4 to 14 from the injury, known as the reparative phase, involves angiogenesis, epithelialisation and provisional ECM formation. The initial stage in the reparative phase is synonymous with healing seen in other tissues, for example, skin and soft tissue. Activated platelets produce EGF and TGF-α; cytokines which stimulate chemotaxis and epithelial proliferation (Grotendorst *et al.*, 1989; Marsell and

Einhorn, 2011). This is relevant in fracture healing where there is simultaneous epithelial damage to surrounding tissue, especially in compound fractures. In this case, epithelialisation actually occurs immediately after injury and is initially stimulated by inflammatory messengers via the up-regulation of keratinocyte growth factors (KGFs) by IL-1 and TNF-α. Fibroblasts synthesise and secrete KGF-1, KGF-2 and IL-6, which stimulate neighbouring keratinocytes to migrate to the wounded area, where they proliferate and differentiate in the epidermis . It would appear that KGF-2 has a substantial influence in orchestrating this process (Jimenez and Rampy, 1999; Sorg *et al.*, 2009).

During the reparative phase of fracture healing, it is both the MSCs and endothelial cells that are the most proliferative. Endothelial cells residing in venules are recruited by VEGF for the redevelopment of capillary tubes. The VEGF is secreted mainly by keratinocytes, but also by macrophages, fibroblasts, platelets and other endothelial cells. Keratinocytes are able to express VEGF through stimulation by IL-1, TNF-α, TGF-β1 and KGF. Endothelial nitric oxide synthase (eNOS) is derived from endothelial cells in response to hypoxia, a process which further increases the production of VEGF. Raised levels of NO initiate vasodilatation and, thus, can protect the new tissue from the toxic effects of ischaemia and reperfusion injury at the fracture site (Witte and Barbul, 2002; Goldman, 2004).

Although the haematoma is a well organised structure, it does not have a significant mechanical role in the immobilisation of the fracture, but instead acts principally as a fibrin scaffold over which repair cells perform their function.

During this phase, the microenvironment of the fracture becomes acidic, which provides an additional stimulus to cellular activity during the early period of fracture healing. However, later in the repair process, the pH gradually reverts to neutral and then corrects to a slightly alkaline level, typically 7.4 (Keramaris *et al.*, 2008).

The cells directly involved in fracture repair are of mesenchymal origin, are pluripotent and are thought to originate from cartilage and bone of the fracture periphery. Some cells originate from the cambium layer within the periosteum, forming the earliest bone, which is particularly active and important in children. Cells derived from the endosteum also participate. Small variations in the fracture microenvironment and the stresses to which they are subjected to, likely determines their fate in terms of cell type differentiation. Surviving osteocytes are not involved in the repair process, as they are destroyed during resorption. However, the majority of cells that are engaged in fracture healing enter the milieu with the granulation tissue, which is composed of new connective tissue and tiny blood vessels, which infiltrates the region from surrounding vessels and is associated with the ingress of capillary buds (Kitaori *et al.*, 2009).

The vascular bed of an extremity undergoes expansion following a fracture, but the osteogenic response is, in the main, confined to the area surrounding the fracture site. Under normal circumstances, it is the periosteal vessels that donate the majority of capillary buds, with the nutrient medullary artery having a more important role later in the healing process. During operative reduction and fixation of the fracture, the surgeon may obstruct this natural process, either by excessively stripping the periosteum, or by destroying the intramedullary system

via the use of nails, screws and plates. In this case, the repair must continue with vessels derived from the surviving tissue (Fayaz *et al.*, 2011).

Mesenchymal stem cells invade the haematoma and begin to rapidly synthesise the soft callus, which is chiefly composed of fibrous tissue and cartilage. This quickly envelopes the fractured bone ends and gives rise to a steady increase in stability of any loose bone fragments. Deviations in oxygen tension lead to the formation of either bone or cartilage, with cartilage being formed in areas in which oxygen tensions are relatively low and vice versa for bone; the ensuing process dependant on the distance of the cell from its blood supply. The developing bridging callus is regulated by FGF and angiopoietins 1 and 2 initially, with VEGF occurring later, during endochondral bone formation (Lieberman *et al.*, 2002; Gerstenfeld *et al.*, 2003; Marsell and Einhorn, 2011).

The cartilage produced is ultimately resorbed by a process similar to endochondral bone formation (discussed later), but is one which is less organised. Bone will be formed, through *primam intentionem*, by cells that receive sufficient oxygen and those which are subjected to the appropriate mechanical stimuli. In early fracture, cartilage formation prevails and glycosaminoglycans exist in elevated concentrations. In the latter stages, the formation of bone becomes more apparent, during which time biochemical events continue with an accumulation of calcium hydroxyapatite crystals. The collagen content returns to a normal level after mineralisation has taken place (Gerstenfeld *et al.*, 2006).

Mineralised tissues are highly organised in their internal structure as a result of specialised cellular activity. The initial step of mineralisation is the formation of

tropocollagen by osteoblasts, which moves from inside the cells to outside, before polymerising to develop collagen fibrils. The collagen fibrils possess their own internal structures, known as hole zones, which are located within the core of the fibrils. They are organised in a regular fashion as a result of the internal structure of the collagen molecules. The initial deposition of mineral occurs in this region due to interactions between metastable solutions of calcium and phosphate and groups of specific amino acid side chains within the holes. This arises due to a series of ordered collagen fibrils, around which there are clustered crystals of calcium hydroxyapatite (Dimitriou *et al.*, 2005; Marsell and Einhorn, 2011; Dimitriou and Giannoudis, 2013).

At this stage, the fractured bone ends gradually become encased in a fusiform mass of callus composed of increasing amounts of bone. Immobilisation of the fragments is more rigid because of the internal and external callus production and clinical union is said to have taken place. However, complete union does not exist at this point, since the remodelling phase begins in the middle of the reparative phase, with resorption of excess or inefficient fragments of the callus and the laying down of trabecular bone along lines of stress (Gerstenfeld *et al.*, 2006; Marsell and Einhorn, 2011).

1.3.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells are non-haematopoietic stromal cells that are fibroblast-like and spindle-shaped in their morphology; these are the essential ‘orchestrators’ of mammalian healing. They are key producers of cytokines that are instrumental

in the inflammatory response and have an important role in the formation of granulation tissue. Chemokines, for example, which are vital in attracting neutrophils and other leukocytes to the wound area, are secreted by these cells (Wong and Martins-Green, 2004). They also have the capacity to become differentiated into chondrocytes, osteoblasts and ultimately osteocytes, which are crucial for the regeneration of bone in the fracture healing process. A study found that fibroblast-like, mesenchymal cells isolated from human tibial fracture haematomas could be differentiated into osteocytes, chondrocytes and adipocytes *in vitro*, thus, having progenitor capabilities (Oe *et al.*, 2007).

Mesenchymal stem cells are identified by their immunophenotype, or expression of various cell surface antigens, which include, amongst others, CD29, CD 44, CD73, CD105 and CD166 in adult humans (for summary, see Table 4) (Haynesworth *et al.*, 1992; Conget and Minguell, 1999; Le Blanc *et al.*, 2003; Sordi *et al.*, 2005). They lack expression of the haematopoietic stem cell markers CD34, CD45 and CD14 (Chamberlain *et al.*, 2007). The ability of MSCs to self-renew and differentiate into a wide range of cell types, notably bone, adipose and cartilage, renders them suitable for use in tissue bio-engineering (Pittenger *et al.*, 1999; Lee *et al.*, 2009; Cartmell *et al.*, 2011). The cells have a migratory capability and systemically-transplanted cells have been shown to migrate to the injury site, conveniently *homing* to tissues that are inflamed or damaged (Barbash *et al.*, 2003).

Table 4: A Summary of Mesenchymal Stem Cell Biochemical Markers

MSC Marker	Alternative Nomenclature	Function
CD29	Integrin β1	Cell adhesion molecule (Haynesworth *et al.*, 1992).
CD44	HCELL	Cell adhesion, migration, 'bone homing receptor' (Haynesworth *et al.*, 1992).
CD54	Inter-cellular Adhesion Molecule1 (ICAM-1)	Cell adhesion and migration (Pittenger *et al.*, 1999).
CD71	Transferrin receptor	Maintains cellular iron homeostasis (Pittenger *et al.*, 1999).
CD73	5'-nucleotidase, ecto (NT5E)	Marker of lymphocyte differentiation (Sordi *et al.*, 2005).
CD90	Thy-1	Cell adhesion, extravasation & migration (Sordi *et al.*, 2005).
CD105	Endoglin	Part of TGF-β receptor complex. Involved in cytoskeletal organisation, cell morphology and migration (Pittenger *et al.*, 1999).
CD106	Vascular Cell Adhesion Molecule-1 (VCAM-1)	Cell adhesion molecule (Conget and Minguell, 1999).
CD166	Activated Leukocyte Cell Adhesion Molecule (ALCAM)	Tissue growth and cell migration (Conget and Minguell, 1999).
Stro-1	-	Adherence and cell proliferation. Identifies bone marrow stromal cell precursors (Galmiche *et al.*, 1993).

1.3.3 Maturation or Remodelling Phase

Regarded as a key phase clinically and the most long-lasting, the maturation and remodelling stage of fracture healing occurs from around day 8 and can continue for up to a year and beyond, depending on the severity of the injury and the healing abilities of the patient. The main aspect of this element is the deposition of collagen and calcium, providing a strong, well-coordinated framework. The strength of a wound may be notably compromised if patients have mineral deposition problems due to malnutrition, disease, alcoholism and other lifestyle factors. Indeed, problems can also arise as a result of tobacco smoking and this is discussed later (Sloan *et al.*, 2010; Patel *et al.*, 2013).

Fracture repair is completed during the maturation phase, when the healing bone is restored to its original shape, structure and mechanical strength. Remodelling of the bone takes place gradually during several months and years and is a process enabled due to mechanical stresses being placed upon the bone. As the fracture site is exposed to an axial loading force, bone is generally laid down where it is needed and resorbed from where it is not. Sufficient strength is typically achieved in 3 to 6 months and then undergoes normal skeletal remodelling thereafter (Dimitriou *et al.*, 2011).

1.3.3.1 Bone Remodelling

The following schematic diagram (Figure 11) illustrates the various stages of the biochemical pathway of the bone remodelling cycle.

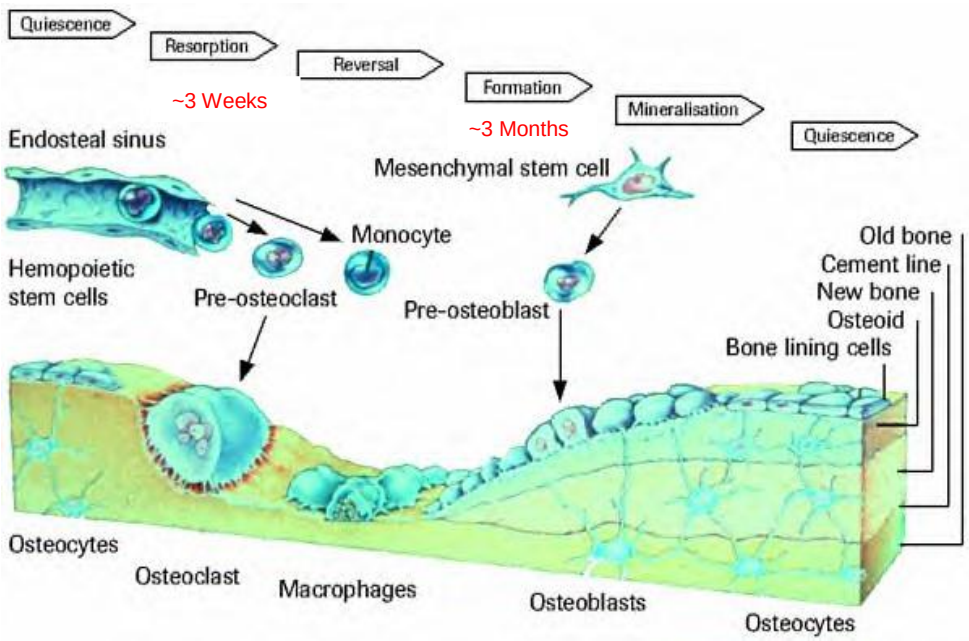

Figure 11: Bone Remodelling Cycle

Image adapted from Jones (2013)

Available from: http://graduatehistology.blogspot.co.uk/

The biochemical resorption and formation of bone occurs simultaneously at different points along the groove or tunnel (Figure 11) (Jones, 2013). Multinucleated osteoclasts resorb bone at the groove's apex in response to increased RANK-L (ligand for receptor activator of nuclear factor kappa B) and M-CSF (macrophage colony stimulating factor) and reduced osteoprotegerin from adjacent osteoblasts. Activation is effected by hormones, cytokines or by osteocytes under mechanical strain. Macrophages appear in the osteoclasts' wake, clear apoptotic cells and prepare the resorbed bone surface as a 'cement line'.

Growth factors released from the bone stimulate differentiation of local stem cells to osteoblasts that form new bone, completing the cycle. The growth factors TNF (tumour necrosis factor), TGF-β (transforming growth factor-beta) and various cytokines (interleukins IL-1, 3, 6, 11) are also involved in this process (Dimitriou *et al.*, 2005).

Osteocytes communicate mechanical strain to osteoblasts on the bone surface via cytoplasmic projections. The osteoblasts respond to this or to other signals such as parathyroid hormone (PTH), dihydroxy-vitamin D_3, cytokines and growth factors by increasing RANK-L and M-CSF and reducing osteoprotegerin. Osteoclast formation ensues from circulating monocyte precursors, responsible for the direction of osteoclasts to the area of bone to be resorbed. Inhibitory signals limit osteoclast recruitment and activity. Macrophages remove apoptotic osteoclasts and prepare the resorbed surface. Growth factors drive osteoblast formation from local stem cells (coupling of resorption and formation), producing osteoid, which is mineralised to form new bone. On completing the cycle (about 120 days), remodelling is balanced if there has been no net change in the amount of bone (Dimitriou *et al.*, 2005).

1.3.3.2 Mineralisation of Bone

The schematic diagram below (Figure 12) portrays events involved in the mineralisation of osteoid to form mineralised bone. Active cuboidal osteoblasts secrete osteoid collagen and matrix vesicles. The matrix vesicles are targets for the deposition of hydroxyapatite crystals; the first step in mineralisation. Confluent mineralisation of osteoid collagen supports the glycosaminoglycan matrix (Young and Heath, 2003).

Figure 12: Mineralisation of bone

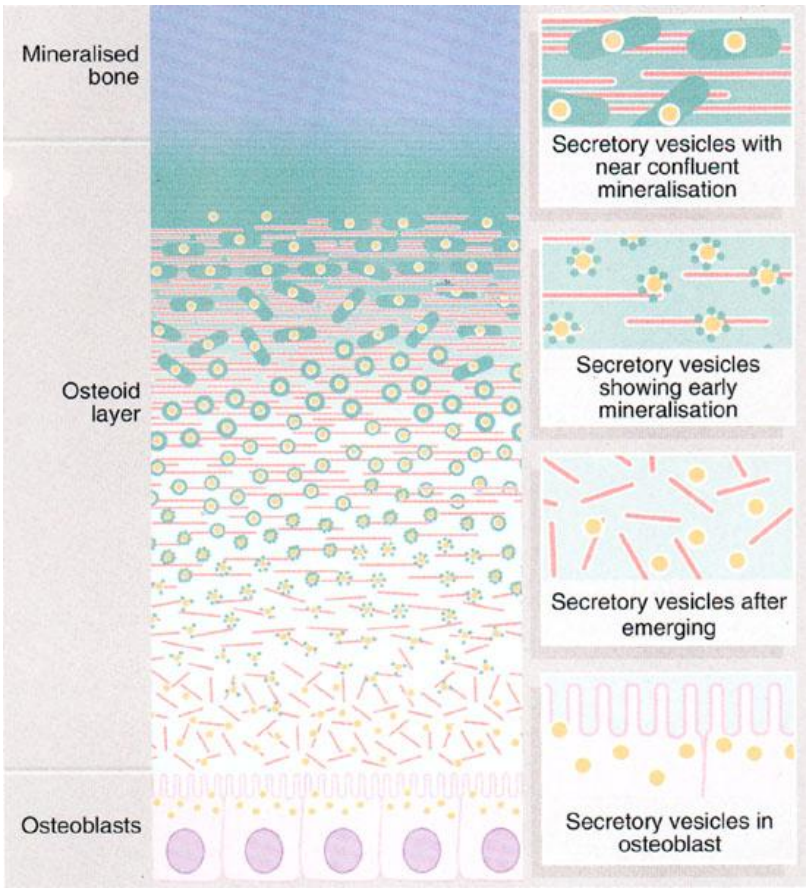

Figure 12: Mineralisation of bone; the matrix vesicles are rich in the enzyme alkaline phosphatase and pyrophosphatase, both of which can produce phosphate ions from an assortment of molecules. The phosphate ions accumulate in the matrix vesicles with calcium ions and together they form hydroxyapatite (Young and Heath, 2003, Pg.183). Image reproduced with permission of Elsevier.

1.3.4 A Summary of the Phases of Fracture Healing Illustrated

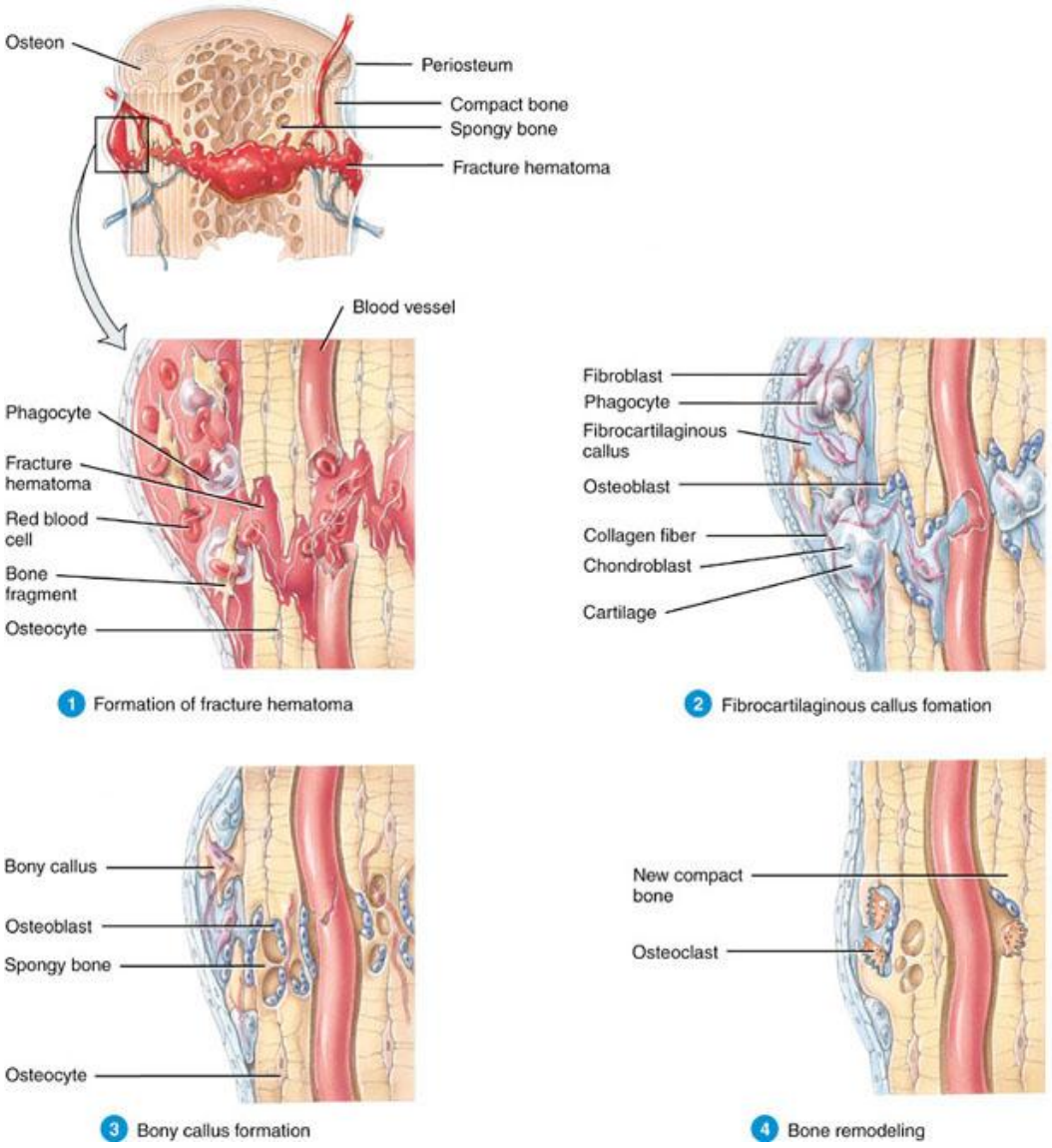

Figure 13: Stages of the Fracture Healing Process.

Figure 13: The diagrammatical stages of fracture healing, from the formation of the fracture haematoma in the inflammatory phase (1), the development of the soft callus (2) to the bony callus (3) in the reparative phase before bone remodelling (4) in the final maturation phase (Tortora and Grabowski, 2003, Pg.176). Image reproduced with permission of John Wiley & Sons, Inc.

Figure 14: The Process of Fracture Healing; Radiology

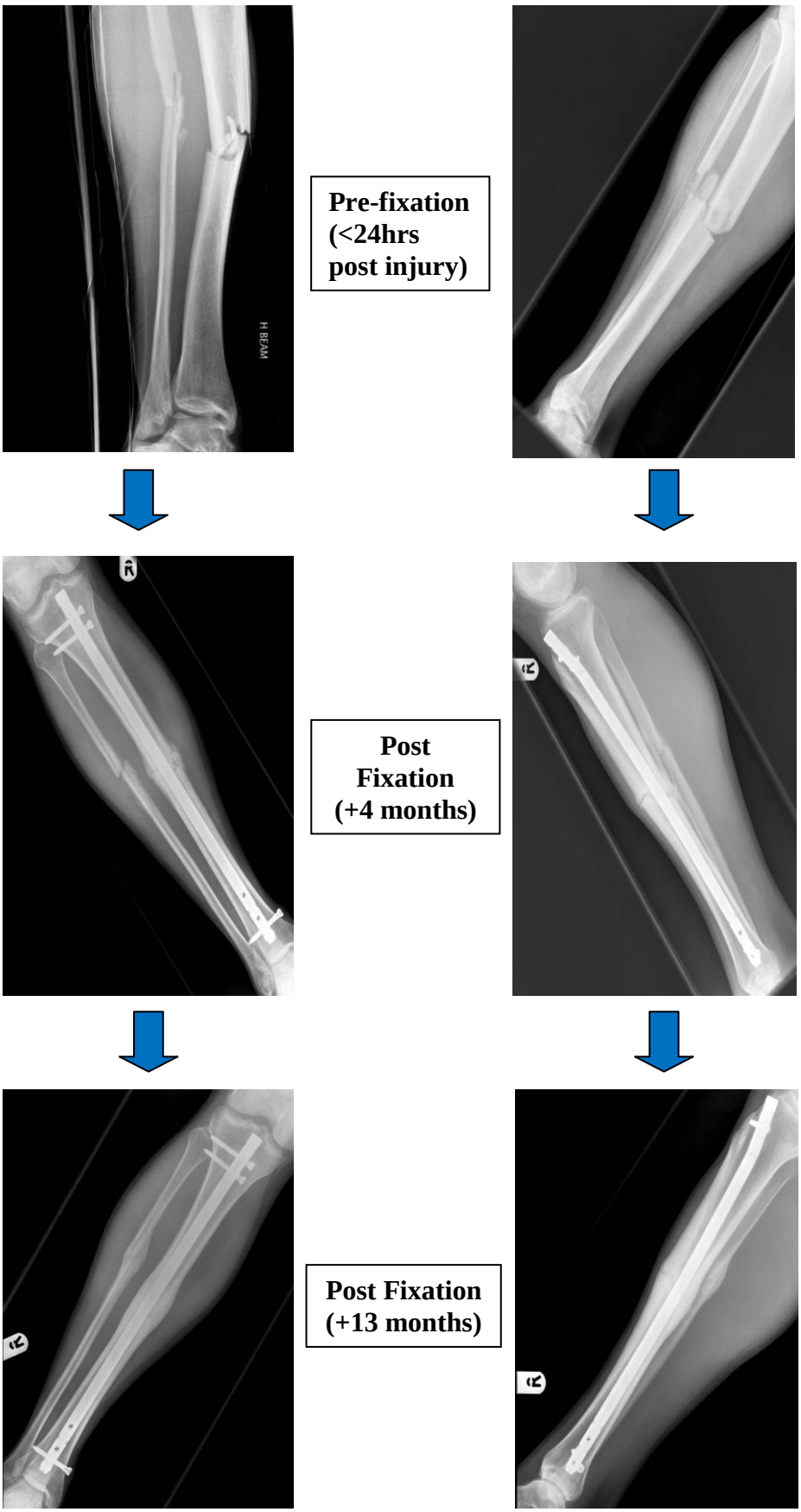

Figure 14 (previous page): The radiographs show the healing progress of a tibia and fibula fracture in the right leg of a 55 year old non-smoking female, who consented to take part in this study. The images were acquired pre- and post-fixation and represent a time-frame of 13 months from the time of injury. The formation of the callus during secondary fracture healing can be observed in the images as endochondral ossification takes place. The patient's healing progress was satisfactory in this particular case.

1.4 Modes of Ossification in Fracture Repair

The basic biology of fracture repair is divided into two parts; direct (primary) and indirect (secondary) fracture healing.

1.4.1 Direct (Primary) Fracture Healing

There are two types of direct or *primary* healing; gap healing and contact healing (see Figure 15). Gap healing takes place after *micrometric* clinical anatomical reduction and rigid fixation, which is usually internal. In the first phase, there is a disruption of the blood supply and necrosis at each of the fracture ends, affecting the bone cellular components, but not necessarily the humoral elements. Even fractures that have been very well surgically reduced have irregular surfaces that can create gaps between fragments. Direct bone formation has the potential to fill gaps of up to 1.0 mm in width and, therefore, removes the requirement for connective tissue and fibrocartilage production prior to bone formation. In the second phase of gap healing, the immature woven bone is longitudinally reconstructed through a process known as Haversian remodelling. Despite effective rigid internal fixation, any gaps greater than l.0 mm are partially filled with fibrous tissue, which is later replaced by bone (Gullihorn *et al.*, 2005).

Contact healing occurs when the fracture fragments are in direct apposition, also known as a 'hairline' fracture, resulting in union through the ingrowth of Haversian systems at the injury site; there is no callus formation in this case (Marshall *et al.*, 2004). Osteoclasts form 'cutting cones' that advance across the fracture line and construct resorption cavities; a process aimed at restoring mechanical continuity. This is followed by the growth of a capillary loop down the centre of the hollow. Mesenchymal stem cells accompany the capillaries and become differentiated into osteoblasts and produce osteoid closing cones. These bone forming units are conical in shape, due to the relative speed of the cutting and closing cones. Osteoclasts are approximately 10 times faster at resorbing bone than the osteoblasts are at its formation and this has important implications on the timing of bone regeneration (Dimitriou *et al.*, 2005) .

Figure 15. Primary Fracture Healing. Radiograph of a fracture of the 5th metatarsal that will likely unify via primary fracture healing. The anterior portion of the fracture is in direct apposition, so will undergo *contact healing*, whilst the posterior aspect shows a small gap of approximately 1.0 mm, whereby *gap healing* is applicable. Image obtained from a consenting patient in this study.

1.4.2 Indirect (Secondary) Fracture Healing

Most fractures heal by *indirect* or *secondary* fracture healing, which results in the formation of a bridging callus through either endochondral (involving cartilage) or intramembranous ossification (not involving cartilage) (discussed later). Bone does not 'evolve' from previously formed cartilage (where applicable), but is instead directly formed via intramembranous ossification. The early phase of secondary fracture healing involves MSCs and provides an early bridging callus, histologically characterised as a *soft callus*. The function of the soft callus is to stabilise the fracture fragments following injury (Dimitriou *et al.*, 2005). Secondary fracture healing, therefore, involves a contribution from the bone marrow during the early phase, when endothelial cells transform into polymorphic cells, expressing an 'osteoblastic phenotype' (Claffey *et al.*, 2001; Marsell and Einhorn, 2011).

The process of secondary fracture healing was first described by McKibbin in 1978 as a five-stage process; haematoma formation, inflammation, soft then hard callus production and finally remodelling (McKibbin, 1978). The stages of fracture healing (applicable in endochondral ossification as discussed later) are now more comprehensively described as an initial haematoma formation with inflammation, angiogenesis and production of cartilage, cartilage calcification, cartilage removal, bone generation and bone remodelling. During the final stage, cartilage becomes calcified and replaced by bone. This developmental process can be seen in the x-ray images as depicted in Figure 14. It is a process enhanced by micromotion, but one which is inhibited by rigid fixation (Marshall *et al.*, 2004; Dimitriou *et al.*, 2005).

1.4.2.1 Endochondral Ossification in Indirect Fracture Healing

This type of bone formation is more common than intramembranous ossification, as it occurs in a mechanically less-stable fracture environment, normally associated with long bone fractures that have been stabilised with plaster of Paris (Ford *et al.*, 2004). The process takes place predominantly adjacent to the fracture site periosteum and is assisted by the soft tissues surrounding the fracture site. Mesenchymal stem cells are recruited to the fracture milieu and subsequently proliferate. They then differentiate into chondroblasts in a process known as chondrogenesis, forming the soft, fibrocartilaginous callus. New chondrocytes synthesise and secrete a cartilage-specific matrix, composed of type II collagen and proteoglycans. After mechanical stability has been achieved, the cartilage matrix is subjected to hypertrophic activity and mineralisation in a spatially organised manner (Gerstenfeld *et al.*, 2003). Following this repair process, vascularisation of the new bone occurs, the calcifying hypertrophic chondrocytes are removed via chondroclastic activity and the formation of woven bone takes place. Apoptosis of chondrocytes follows ossification of the callus as the former is replaced by the woven marrow-filled bone. Substantial remodelling takes place in order for the newly-formed bone to become stable and able to weight-bear (Dimitriou *et al.*, 2005; Marsell and Einhorn, 2011).

1.4.2.2 Intramembranous Ossification in Indirect Fracture Healing

Intramembranous ossification takes place where fractures have been surgically reduced and stabilised using metal plates and screws and involves the laying down of bone into the mesenchyme. The sources of the cells that are relevant in intramembranous formation of bone are provided by the following;

- Underlying cortical bone
- Proximal periosteum
- Bone marrow

Twenty four hours following the onset of the fracture, local MSCs migrating from the bone marrow become differentiated into an osteoblastic phenotype. Seventy two hours following fracture, cortical osteoblasts and osteoprogenitors (the latter derived from periosteal cambium) divide and differentiate, forming a hard callus, or woven bone that envelopes the fracture site. The proliferation of these cells peaks at between 7 and 10 days and, despite continued activity, is substantially decreased after 14 days (Gerstenfeld *et al.*, 2003).

Intramembranous ossification is the simpler method of bone formation and differs from endochondral ossification in that cartilage is not present in this process. The flat bones of the skull and mandible are formed via intramembranous ossification during foetal development. During this type of bone development in bone healing, mesenchymal stem cells cluster at the site of the fracture and differentiate into osteogenic cells and then into osteoblasts. The site of the cluster is known as the 'centre of ossification', where osteoblasts secrete the organic matrix of the

bone until they become surrounded by it. There then follows a calcification stage, when the secretion of the matrix ceases and the cells become osteocytes. After several days, Ca^{2+} is deposited and the matrix becomes calcified and hardens. As the bone matrix forms, it develops into trabeculae and blood vessels grow along the spaces between this spongy bone and mesenchyme along the surfaces of the newly formed bone. The connective tissue that is associated with vascularisation differentiates into bone marrow. The development of periosteum then ensues, which occurs at the periphery of the bone from the condensed trabeculae. A thin layer of compact bone then forms, which covers the surface layers of spongy bone prior to remodelling (Tortora and Grabowski, 2003).

1.5 MOLECULAR ASPECTS OF FRACTURE HEALING

As with all repair processes, fracture healing is initiated and regulated by the immune system. There is an established association between the immune system and the skeleton; this has been well recognised since the 1970s, when several seminal studies were published (Horton *et al.*, 1972; Mundy *et al.*, 1974; Horowitz *et al.*, 1984; Dewhirst *et al.*, 1985). Current evidence also indicates that there is a close interrelationship between the biology of bone and host defences, giving rise to the term *osteoimmunology*. This represents a research area that focuses on the biomolecular interactions of both the cellular and molecular components (Arron and Choi, 2000; Dimitriou *et al.*, 2005).

The inflammatory and anti-inflammatory molecular mediators are those soluble factors of the immune system that are expressed in and secreted by cells, platelets and leukocytes in order to control damage and maintain an efficient repair process. There are three categories of *promoter molecules* involved in early fracture healing; (i) the pro-inflammatory cytokines, (ii) the TGF-β superfamily and (iii) the metalloproteinases and angiogenic factors (Gerstenfeld *et al.*, 2003). The other significant mediators involved in healing are growth factors, chemokines, lymphokines and monokines, eicosanoids, prostaglandins, lipoxins, kinins and cellular metabolites (Oberyszyn, 2007). These humoral intermediaries of fracture healing and associated immune response are discussed here.

1.5.1 Proinflammatory Cytokines

Cytokines are a group of intracellular and extracellular proteins and peptides that are used in organisms as chemical signalling agents, allowing cells to communicate with each other. The cytokine family consists of small water-soluble proteins and glycoproteins with a mass typically between 5 and 30 kilodaltons (kDa). Cytokines are clinically important in both innate and adaptive immune responses. Due to their central role in the immune system, cytokines participate in a variety of immunological, inflammatory and infectious diseases; they are also involved in several developmental processes during embryogenesis. Cytokines can be subcategorised as chemokines, lymphokines, monokines, interleukins, colony-stimulating factors and interferons (Mlodzikowska-Albrecht *et al.*, 2007).

The cytokines interleukin-1, IL-6 and TNF-α have a key role in initiating the healing process of fractured bones (Gerstenfeld *et al.*, 2003). They are referred to as positive acute-phase proteins and are secreted by macrophages, inflammatory cells and also cells of mesenchymal origin that are found in the periosteum. Their function is to promote chemotaxis, thereby attracting other inflammatory cells to the site of injury. This mechanism stimulates extracellular matrix synthesis, angiogenesis and the recruitment of endogenous fibrogenic cells to the affected area. There is peak concentration of these proteins within the first 24 hours of the fracture occurring, followed by a drop in levels during the period of cartilage formation. Cytokine quantities are later restored and become markedly increased again during the remodelling phase (Gerstenfeld *et al.*, 2003).

The formation and remodelling of endochondral bone is also regulated by cytokines (Gerstenfeld *et al.*, 2003). For example, TNF-α stimulates the migration of MSCs, evoking apoptosis of hypertrophic chondrocytes and stimulating osteoclastic activity. Indeed, absence of TNF-α causes delayed resorption of mineralised cartilage, thereby inhibiting bone formation. Levels of IL-1, IL-6 and TNF-α are increased again during callus reshaping in the latter stages of fracture healing and remodelling (Uchida *et al.*, 2003; Dimitriou *et al.*, 2005). Importantly, there are temporal aspects associated with the activity of growth factors. For example, IL-1 and IL-6 peak at 24 hours post-fracture, but are barely detectable after three days (Lee *et al.*, 2004). The table overleaf summarises the interleukins (IL-1-IL-10) that are involved in the inflammation stage of fracture healing.

Table 5: Inflammatory Interleukins 1to10 (IL-1 to IL-10) in Fracture Repair

INTERLEUKIN	ROLE	SOURCE
IL-1	Induces fever, ACTH release, enhances TNF-α, IFN-γ; activates granulocytes, endothelial cells, stimulates haematopoiesis.	Macrophages, mast cells, keratinocytes, lymphocytes.
IL-2	Activates macrophages, T lymphocytes, NK cells, stimulates differentiation of B lymphocyte; induces fever.	Macrophages, mast cells, keratinocytes, T lymphocytes.
IL-3	Stimulates bone marrow stem cells	T lymphocyte
IL-4	Early: Stimulates fibroblast proliferation; Late: Downregulates cytokine expression	Mast cells.
IL-5	B-cell differentiation, eosinophil and IgA production	TH2 lymphocytes
IL-6	Released in response to IL-1; induces fever; enhances release of acute-phase reactants by the liver; inhibits ECM breakdown during proliferation.	Macrophages, mast cells, keratinocytes, lymphocytes. (pro-inflammatory mediator)
IL-7	Lymphocyte maturation	Stromal cells of red bone marrow and thymus
IL-8	Enhances neutrophil adherence, chemotaxis and granule release; enhances epithelialisation.	Macrophages, mast cells, keratinocytes, lymphocytes.
IL-9	Stimulate mast cells	T lymphocytes
IL-10	Downregulates cytokine expression in latter stages (anti-inflammatory).	Mesenchymal stem cell.

(Cross and Mustoe, 2003)

1.5.2 Transforming Growth Factor Beta (TGF-β)

There are many members of the TGF-β superfamily, consisting of numerous growth and differentiation factors. More than 30 TGF-β superfamily members have been identified in the human genome and include the BMPs, growth differentiation factors (GDFs), activins, inhibins, the Mullerian inhibiting substance, in addition to the various numerical subtypes (i.e., TGF-β1, TGF-β2 and TGF-β3) in humans (Tardif *et al.*, 2004). Transforming growth factor derivatives are produced by the activity of proteolytic enzymes on large proteins (Shimasaki and Ling, 1991) and act upon serine/threonine kinase receptors (Type I and Type II) in target cells (Lind *et al.*, 1991). The ligand-receptor interaction initiates an intracellular signalling pathway that influences gene expression within the nucleus of the cell it is bound to. This then initiates and promotes the various stages of intramembranous and endochondral bone ossification associated with fracture healing (Lee *et al.*, 2004).

1.5.3 TGF-β Cell Signalling Pathway

The TGF-β ligand initiates signalling by binding to and bringing together type I and II receptor serine/threonine kinases located on the cell surface. Receptor II is then allowed to phosphorylate receptor I kinase domain, which then disseminates the signal via phosphorylation of the Smad proteins (Figure 16). There are eight different Smad isoforms, constituting three functional classes; the receptor-regulated Smad (R-Smad), the Co-mediator Smad (Co-Smad) and the Inhibitory Smad (I-Smad). R-Smads (Smad1, 2, 3, 5 and 8) are phosphorylated and

activated directly by the type I receptor kinases and undergo homotrimerisation and formation of heteromeric complexes with the Co-Smad, Smad4. The activated Smad complexes are translocated into the nucleus and, in combination with other nuclear cofactors, control the transcription of target genes. The I-Smad, Smad6 and Smad7, negatively regulate TGF-β signalling by competing with the R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation (Shi and Massague, 2003).

Figure 16: TGF-β Cell Signalling Pathway

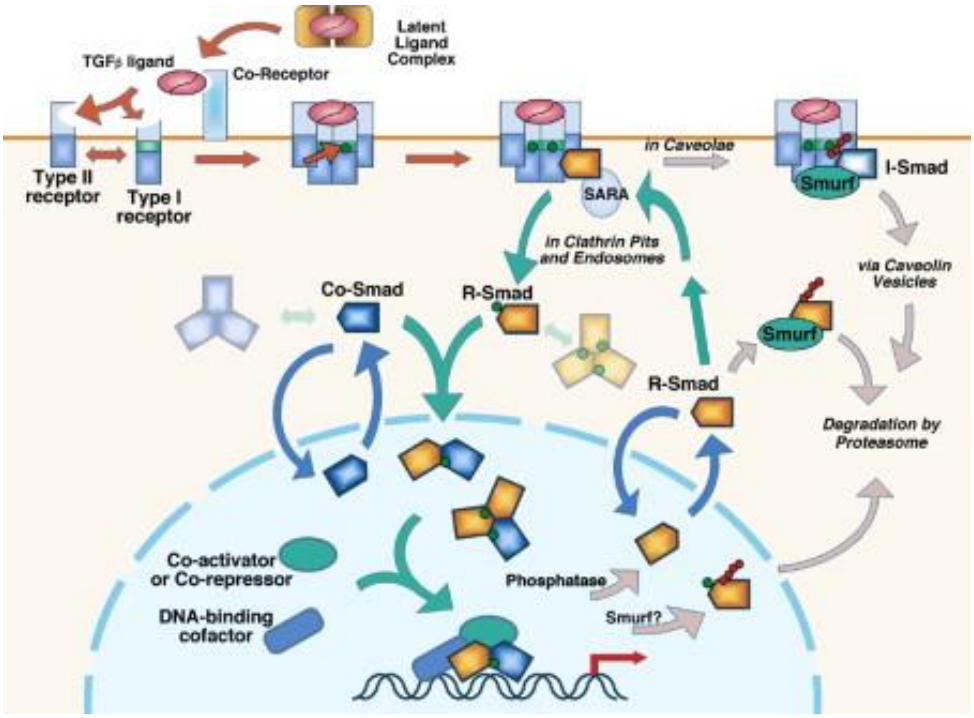

Figure 16: TGF-β signalling from cell membrane to the nucleus. The arrows demonstrate signal flow. Orange arrows represents ligand and receptor activation; grey represents Smad and receptor inactivation; green arrows, Smad activation and formation of a transcriptional complex; blue arrows, Smad nucleocytoplasmic shuttling. Also depicted are phosphate groups (green circles) and ubiquitin (red circles) (Shi and Massague, 2003). Image reproduced with permission of Elsevier.

1.5.4 Subtypes of Transforming Growth Factor – Beta (TGF-β)

In mammalian subjects, there are three subtypes of TGF-β that are involved in healing; TGF-β1, TGF-β2 and TGF-β3. These are released by platelets during the inflammatory phase of fracture healing; they are also secreted by macrophages, monocytes and lymphocytes in normal, non-traumatised tissues. The TGF-βs are believed to be involved in the early stages of callus formation (Connor and Evans, 1982; Kingsley, 1994; Bostrom, 1998). In addition to platelets and leukocytes, TGF-β is produced by osteoblasts and chondrocytes and is stored in the bone matrix (Lieberman *et al.*, 2002). It is a ligand that binds to a kinase receptor, activating intracellular pathways (Simonsson *et al.*, 2005). This growth factor is a large cytokine that is capable of mobilising acute phase proteins via chemotaxis and therefore promotes the proliferation of MSCs, pre-osteoblasts, chondrocytes and osteoblasts (Lieberman *et al.*, 2002). There is an additional influence on the production of extracellular proteins such as collagen, proteoglycans, osteopontin, osteonectin and alkaline phosphatase (Sandberg *et al.*, 1993; Zimmermann *et al.*, 2007).

It has been suggested that TGF-β2 and TGF-β3 have more important roles than TGF-β1 in fracture healing, as expression of isoforms two and three tend to peak during chondrogenesis. There is a high basal level of TGF-β1 in healthy diaphyseal bone that remains unchanged in the event of a fracture, possibly rendering this type less significant than TGF-β2 and TGF-β3 (Lee *et al.*, 2004). Moreover, there is controversy regarding the therapeutic potential for exogenous

TGF-β in terms of enhancing bone repair (Lieberman *et al.*, 2002; Zimmermann *et al.*, 2007).

1.5.5 Bone Morphogenetic Proteins (BMPs)

Bone morphogenic proteins are pleiotropic morphogens that are capable of regulating growth, differentiation and apoptosis of osteoblasts, chondroblasts, neural cells and epithelial cells (Sakou, 1998). The production of BMPs involves various ECM-based factors and includes osteoprogenitors, mesenchymal stem cells, osteoblasts and chondrocytes; BMPs are essential in fracture healing because they elicit a series of events relevant to chondro-osteogenesis. This typically features chemotaxis, mesenchymal (and osteoprogenitor) cell proliferation and differentiation (Sammons *et al.*, 2004), angiogenesis and ECM synthesis (Prisell *et al.*, 1993; Yu *et al.*, 2010). There are four subtypes of BMPs, categorised according to their primary amino acid sequence and members are categorised into groups as follows;

- Group 1: BMP-2, BMP-4,
- Group 2: BMP-5, BMP-6, BMP-7,
- Group 3: GDF-5 (BMP-14), GDF-6 (BMP-13), GDF-7 (BMP-12),
- Group 4: BMP-3 (osteogenin), GDF-10 (BMP-3b)

(Dimitriou *et al.*, 2005)

There is a structural and functional similarity amongst BMPs, although each subtype has a unique role within fracture healing. The BMPs also have an ability

to stimulate the synthesis and secretion of other bone repair factors such as insulin-like growth factor (IGF) and VEGF (Deckers *et al.*, 2002; Keramaris *et al.*, 2008). It may be worth noting that BMP-1 is *not* a member of the TGF-β superfamily. It is classified as a metalloproteinase that is capable of cleaving the BMP-2 antagonist chordin and is involved in the direction of bone and cartilage formation *in vivo*. It is for this reason that the protein was first thought to behave like other BMPs (Nelson *et al.*, 1994; Bond and Beynon, 1995; Wozney, 2002; Hartigan *et al.*, 2003).

Table 6: Temporal and Functional Characteristics of TGF-β in fracture healing

TGF-β member	Time of expression (from point of injury)	Function
GDF-8	Day 1 only	Negative regulator of skeletal muscle growth
BMP-2	Day 1 to 21	Recruitment of MSCs, chondrogenesis, role in fracture healing cascade, regulates expression of BMPs; differentiation of MSCs
BMP-3, 8	Day 14 to 21	Regulation in osteogenesis
BMP-4	Day 14 to 21	Involvement in early stage of callus formation
BMP-7	Day 14 to 21	Regulation of ossification
GDF-10 BMP-5, 6	Day 3 to 21	Regulation in ossification, BMP-6 initiates chondrocytes maturation
GDF-5, 1	Day 7 to 14	GDF-5 involved in chondrogenesis; Stimulation of MSC aggregation, angiogenesis, chemotaxis, degradation of matrix proteins
TGF-β1 TGF-β2 TGF-β3	Day 1 to 21 Day 3 to 14 Day 3 to 21	Chemotactic for bone forming cells and macrophages; proliferation of MSC, osteoblasts, chondrocytes

(Russell *et al.*, 1980; Dong and Canalis, 1995; Cho *et al.*, 2002; Devlin *et al.*, 2003; Aspenberg, 2013)

1.5.6 Metalloproteinases and Angiogenesis

Successful bone repair requires sufficient blood flow to the injury site. In the final phases of bone healing, there is endochondral ossification and bone remodelling. During this remodelling phase, matrix metalloproteinases degrade cartilage and bone, allowing for the permeation of blood vessels. The VEGF-dependent and angiopoietin-dependent pathways are believed to be mediators in angiogenesis and are, therefore, highly active in the fracture repair process (Gerstenfeld *et al.*, 2003). There are two forms of angiopoietin; angiopoietin I and II, both of which are regulatory vascular morphogenic molecules related to the formation of larger vessels and development of collateral branches of existing vessels. Exogenous administration of VEGF has been shown to improve fracture repair (Street *et al.*, 2002; Keramaris *et al.*, 2008).

1.5.7 Growth Factors

The term 'growth factor' applies to a group of proteins, weighing between 4 and 60 kDa that are capable of stimulating cellular proliferation and cellular differentiation. Growth factors are vital for regulating a variety of cellular processes and act as cell signalling molecules. Examples are cytokines and hormones that bind to specific receptors on the surface of their target cells. They promote cell differentiation and maturation, which varies between different growth factors. For example, bone morphogenetic protein (BMP) stimulates bone cell differentiation, while vascular endothelial growth factor (VEGF) stimulates blood vessel formation and differentiation. For the circulatory system and bone

marrow in which cells are free and unbound, it is preferable for them to communicate by soluble, circulating protein molecules (Cross and Mustoe, 2003).

While 'growth factor' implies a positive effect on cell size, 'cytokine' is perhaps a neutral term with reference to whether a molecule affects proliferation. In this sense, some cytokines can be growth factors, such as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF). However, some cytokines have an inhibitory effect on cell growth or proliferation. Yet others, such as Fas ligand, are used as 'death' signals, as they cause target cells to undergo apoptosis. Cytokines can affect cellular function through endocrine, paracrine, autocrine and intracrine mechanisms (Cross and Mustoe, 2003; Goldman, 2004).

Individual growth factor proteins tend to be derivatives of members of larger families of structurally and evolutionary-related proteins. There are many growth factor families, e.g., VEGF, transforming growth factor-beta (TGF-β) (of which BMP is a member), neurotrophins (nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin$_3$ (NT_3)) and fibroblast growth factor (FGF) (Shimasaki and Ling, 1991; Beer *et al.*, 2000; Cross and Mustoe, 2003). Most growth factors are derived from larger proteins that have been the focus of post-translational modification before being expressed in an active state. Growth factor receptors are transmembrane glycoproteins that are activated through tyrosine kinase enzyme and phosphorylation reactions, as illustrated in Figure 16 (Bennett and Schultz, 1993; Shi and Massague, 2003).

Table 7: Growth Factors of the Immune System

GROWTH FACTOR	FUNCTION
Vascular Endothelial Growth Factor (VEGF)	Mitogen for vascular endothelial cells in angiogenesis (Petreaca *et al.*, 2008).
Transforming growth factor beta (TGF-β)	Mitogenic or antiproliferative depending on cell type; regulates ECM formation. Anti-inflammatory effect in healing (Frangogiannis, 2008).
Granulocyte-colony stimulating factor (G-CSF)	Proliferation/differentiation stimulator in neutrophils and granulocytes (Seshadri *et al.*, 2008).
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Pleiotropic stimulator and activator of granulocyte and monocyte proliferation (Sugano *et al.*, 2005).
Nerve growth factor (NGF)	Differentiation of sensory, cholinergic and sympathetic neurons (Madduri *et al.*, 2009).
Neurotrophins (NT-3, 4, 5 & 6)	Differentiation/survival of sensory neurons (Madduri *et al.*, 2009).
Platelet-derived growth factor (PDGF)	Mitogenic and differentiating activity in endothelial cells and neurons (Lin *et al.*, 2009).
Erythropoietin (EPO)	Stimulates proliferation of erythroid progenitors; induces haemoglobin production (Sorg *et al.*, 2009).
Thrombopoietin (TPO)	Promotes megakaryocyte development (Huang *et al.*, 2009).
Transforming growth factor alpha (TGF-α)	Mediator of bone resorption and wound healing (Leker *et al.*, 2009).
Epidermal growth factor	Mitogenic and differentiating in many cell types (Borisov *et al.*, 2009)
Basic fibroblast growth factor (bFGF or FGF2)	Mitogenic activity in embryonic and adult tissues and tumour cells (Sun *et al.*, 2009).
Insulin-like growth factor	Mediator of growth hormone activity and cell development (Steigen *et al.*, 2009).
Hepatocyte growth factor (HGF)	Mitogenic activity in hepatocytes. Stimulator of motility and matrix invasion of epithelial cells (Liu *et al.*, 2006).

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1.5.8 Platelet-Derived Growth Factor (PDGF)

Platelet derived growth factor (PDGF) is a dimeric polypeptide composed of α and β chains; it is a ligand of the tyrosine kinase receptor and its binding is affected by IL-1, TNF-α and TGF-β1 (Solheim, 1998). It is chemotactic for MSCs and is synthesised by platelets, monocytes, macrophages, endothelial cells and osteoblasts (Andrew *et al.*, 1993). There are potentially beneficial clinical implications for PDGF and Nash *et al.*, (1994) showed that PDGF increased callus density and volume in the tibial fractures of rabbits (Nash *et al.*, 1994; Tsiridis *et al.*, 2007).

1.5.9 Fibroblast Growth Factor (FGF)

There are nine structurally-related polypeptides of the FGF family, which are bound to the tyrosine kinase receptor. In normal adult tissue, FGFs are both acidic *and* basic in nature and there are varying degrees of potency between members. In fracture healing, FGFs are synthesised by monocytes, macrophages, MSCs, osteoblasts and chondrocytes. The growth and differentiation of all of these factors are further promoted by FGFs (Zimmermann *et al.*, 2007; Jemal *et al.*, 2008).

Fibroblast growth factors are active in the initial period of bone repair and are therefore crucial in angiogenesis and MSC mobilisation. There is also an instrumental involvement in chondrocyte proliferation and maturation and evidence suggests that FGF can have an advantageous influence on callus size (Matzuk *et al.*, 1995; Lieberman *et al.*, 2002; Gerstenfeld *et al.*, 2006).

1.5.10 Insulin-Like Growth Factors (IGFs)

Also known as somatomedin-C, IGF-I and IGF-II originate from endothelial cells, osteoblasts and chondrocytes. Serum concentrations of these growth factors are chiefly regulated by growth hormone and their biologic actions are controlled via the cellular mechanisms of IGF binding proteins (IGFBPs) (Shimasaki and Ling, 1991; Solheim, 1998; Lieberman *et al.*, 2002). Insulin-like growth factor encourages bone matrix formation by mature osteoblasts and is more potent than the IGF-II isomer. The latter becomes active at a later stage of endochondral bone formation and promotes type I collagen production, cartilage synthesis and cell proliferation (Prisell *et al.*, 1993; Gerstenfeld *et al.*, 2003).

1.5.11 Colony Stimulating Factor (CSF)

Colony stimulating factor (CSF) is a member of a group of cytokines that stimulate wound-healing. Macrophages secrete CSF-1, an autocrine mediator, in order to 'reproduce' itself in an act of 'self-preservation'. The macrophage releases granulocyte-macrophage CSF, which has chemotactic, as well as cellular proliferation and activation abilities (Tsiridis *et al.*, 2007).

1.5.12 Chemokines

Chemokines represent a subdivision of cytokines that are soluble proinflammatory mediators capable of attracting and activating leukocytes. They are subdivided into four families depending on their specific amino acid formation at the first two cysteine residues closest to the *N* terminus. This factor is important in that the

different cysteine patterns are chemoattractive to different classes of leukocytes (Luster, 1998; Broughton *et al.*, 2006; Heo *et al.*, 2011).

1.5.13 Lymphokines and Monokines

Lymphokines are a subset of cytokines that are produced by activated T lymphocytes. Monokines are produced by mononuclear phagocytes. Interleukins represent a group of cytokines that were thought to be secreted by one type of leukocyte that acts upon a different type of leukocyte. All of these cytokines are expressed by non-haematopoietic cells, or MSCs, providing a range of biological functions. The interleukin (IL) numbering system was devised in 1978 and ranges from IL-1 to IL-36 (Oppmann *et al.*, 2000; Kleiner *et al.*, 2013).

1.5.14 Eicosanoids

Eicosanoids are signalling molecules that originate from omega-3 (ω-3) and omega-6 (ω-6) essential fatty acids. They exert important control of inflammation and immunity and are also cell messengers of the central nervous system (CNS). They are a superfamily of biologically active compounds derived from the oxygenation of arachidonic acid. Specific (and non-specific) stimuli, such as cellular damage, free radicals or osmotic stress, cause the release of phospholipase A_2 (PLA_2) (Boyer *et al.*, 1995; Thoroed *et al.*, 1997). At the cell membrane, PLA_2 hydrolyzes arachidonic acid (generally phosphatidylcholine) to produce arachidonate metabolites. Following this specialised cleavage step, arachidonic acid is swiftly re-esterified into membrane lipids or is bound by intracellular proteins, rendering it unavailable for additional metabolism (Raikin *et al.*, 1998).

In the event that arachidonic acid avoids re-esterification, it becomes freely available as a substrate for further enzymatic conversion. The reaction results in the addition of oxygen atoms at varying sites of the fatty acid backbone, giving rise to biologically active eicosanoids (Fitzpatrick and Soberman, 2001; Kalish *et al.*, 2013).

There are four families of eicosanoids; prostaglandins, prostacyclins, thromboxanes and leukotrienes (Soberman and Christmas, 2003). The prostaglandins (PGs) are mediated by cyclooxygenase 1 and 2 (COX-1 and COX-2) and exist as a series of isoforms; PGD, PGE, PGF, PGG and PGH, with a subscripted numeral that designates the number of double bonds within the compound (Smith, 2013). The PGs most relevant in inflammation are the prostacyclins PGE_2, PDG_2, PGF_2-α, PGI_2 and thromboxane A_2 (TxA_2); each is formed by the activity of a specific enzyme. Thromboxane A_2 is the major product formed by platelets and is both an effective platelet-aggregating agent and vasoconstrictor; it is unstable and is rapidly converted to its inactive form, thromboxane B_2 (TxB_2), a metabolite and not involved in platelet aggregation (Mayer *et al.*, 2005). Prostacyclin synthase, which can be located in the endothelium, is able to assist with the formation of PGI_2 and the more robust isoform, PGF_1-α. Prostacyclin is a vasodilator that inhibits the aggregation of platelets; it is also capable of increasing the chemotactic capacity of other inflammatory mediators (Tilley *et al.*, 2001; Kalish *et al.*, 2013).

1.5.15 Prostaglandins

The prostaglandins contribute to the pathogenesis of inflammatory pain (*dolor*), redness (*rubor*) and fever (*calor*). Prostoglandin E_2 (also known as dinoprostone) causes hyperalgesia of the skin, due to suboptimal concentrations of intradermal histamine and bradykinin. It causes softening of the cervix and stimulates uterine contraction during labour and is also known to motivate the release of certain factors by osteoblasts, which in turn promote bone resorption by osteoclasts. There is a febrile cytokine interaction with PGE_2 to promote fever in the event of infection. The prostaglandins PGE_2, PGD_2 and $PGF_2\alpha$ are the primary metabolites of the cyclooxygenase pathway in mast cells, causing vasodilatation and oedema formation (Dray, 1995; Nelson *et al.*, 2013).

1.5.16 Lipoxins

First described by Serhan *et al* (1984), lipoxins are anti-inflammatory lipid mediators generated from arachidonic acid; transcellular biosynthesis involving platelets and leukocytes is essential for their formation (Serhan *et al.*, 1984). Platelets can form lipoxins from neutrophil-derived intermediates. For example, lipoxins A_4 and B_4 (LXA_4, LXB_4) are formed by the action of platelet 12-lipoxygenase on leukotriene A_4 (LTA_4) in neutrophils (Kilfeather, 2002). Lipoxin production is inhibited by blocking cellular adhesion, but augmented by intricate cell-to-cell contact. This eicosanoid is capable of evoking both pro-inflammatory and anti-inflammatory processes. There is a tendency of lipoxins to inhibit chemotaxis and adhesion in neutrophils, but to cause cellular adhesion in monocytes (Maddox *et al.*, 1998; Chinthamani *et al.*, 2012).

1.5.17 Interferon (IFN)

The interferon (IFN) group is made up of three subtypes; IFN-α, IFN-β and IFN-γ. Each group is named according to their capability to 'interfere' with viral proliferation and survival. Interferon-alpha, for example, is mainly produced by monocytes, macrophages, B lymphocytes and natural killer (NK) cells. It is capable of limiting viral replication within virus-infected cells, preventing contamination of uninfected cells and stimulates anti-viral immunity from cytotoxic lymphocytes and NK cells. Also stimulated are antigen-presentation, cytokine production by monocytes as well as monocyte effector functions such as adherence, phagocytosis and nitric oxide (NO) production. There is a net accumulation of macrophages, in addition to an increase in the destruction of intracellular pathogens. Interferon-gamma also promotes killing by activating NK cells and neutrophils and by stimulating the adherence of granulocytes to endothelial cells via the recruitment of intracellular adhesion molecules, an action that is also undertaken by IL-1 and tumour necrosis factor-alpha (TNF-α) (Farrar and Schreiber, 1993; Franceschetti *et al.*, 2009).

1.6 INHIBITORY MOLECULES INVOLVED IN FRACTURE HEALING

There are various inhibitory molecules that are capable of regulating certain biochemical pathways involved in bone regeneration, maintenance of adult tissues and embryonic development. The inhibition of cellular processes incorporates negative feedback loops and *crosstalk* that will decrease the exposure of cells to signalling molecules, thus affecting their behaviour. Inhibition can take place at any stage of the cascade, whether in the ECM or at the cellular level in the cell membrane, the cytosol or in the nucleus. The fact that inhibition can feature at any stage of the cascade reflects the complexity of signalling regulation that is involved in fracture healing (Dimitriou *et al.*, 2005). Signalling molecules regulate the progression of endochondral and intramembranous bone regeneration and achieve this by recruiting undifferentiated MSCs and osteoprogenitor cells before inducing their differentiation and subsequent proliferation into osteoblasts (Dimitriou and Giannoudis, 2013).

1.6.1 Extracellular Matrix Inhibition

At the ECM level, various molecular inhibitors target BMPs and therefore are termed 'BMP antagonists'. The antagonists that exist in the extracellular compartment become specifically bound to BMPs, altering certain developmental and cellular processes. Specific antagonistic molecules are released into the ECM by osteoblasts, the stimulation of which is increased on exposure to BMPs. This is due to the presence of local autoregulatory feedback mechanisms that occur

during injury, involving the rapid appearance of BMPs at the fracture site (Tsiridis *et al.*, 2007).

1.6.2 Noggin

The secreted protein noggin binds exclusively to BMP-2, -4, -5, -6 and -7 and also GDF-5 and -6. The binding of noggin prevents the bound BMPs interacting with their respective cellular receptors (Gazzerro *et al.*, 1998; Aspenberg, 2013). Osteoblasts express noggin in response to high levels of BMP in the ECM, reducing the effects of BMPs on osteoblastic differentiation and function (Gazzerro *et al.*, 1998; Devlin *et al.*, 2003). The exogenous administration of BMP-2 counteracts this process by inhibiting the actions of noggin, thus increasing osteoblastogenesis and optimising the regulation of callus formation. Experimentally, the addition of noggin antibody (and ultimately its inhibition) can also promote osteoblastogenesis. Indeed, the balance of noggin and another BMP subtype, BMP-4, is important in the regulation of the callus formation in fracture repair and is vital in certain other adult biological processes, including the formation of joints (Russell *et al.*, 1980; Yoshimura *et al.*, 2001; Tsiridis *et al.*, 2007).

Mammalian *in vivo* studies, concerning the role of noggin in terms of skeletal function, found that transgenic mice over-expressing the noggin gene (NOG, locus 17q22) were susceptible to fractures in the developmental stages following birth. This was consistent with a low bone mass, osteopenia and abnormal osteoblastic function. Humans with NOG mutations can present frank skeletal and joint impairment, whilst NOG knockout mice die shortly after birth and have dense, deformed long bones, implicating the mediatory role of noggin (Brunet *et*

al., 1998; Devlin *et al.*, 2003). The rare genetic bone disease fibrodysplasia ossificans progressiva (FOP) is characterised by abnormal hallux development and progressive heterotopic endochondral ossification of the muscles. This disorder is due to a lack of expression of the NOG gene (Fontaine *et al.*, 2005).

1.6.3 Chordin

The antagonist chordin (Chd) binds with BMP-2, BMP-4 and BMP-7 in a similar way to noggin, thereby antagonising the actions of BMP by blocking the binding action to its receptors. Chordin also has a role in embryonic development, especially in skeletogenesis, as high levels are expressed in cartilage and in the epiphyseal regions of developing long bones (Bostrom, 1998; Balemans and Van Hul, 2002; Yang *et al.*, 2013). Expression of Chd *in vitro* has been found to be inversely related to chondrocyte maturation and differentiation due to BMP inhibition and is considered to be a negative regulator of endochondral ossification. The secreted protein *twisted gastrulation* (TSG) is a cofactor that attaches to Chd, thus facilitating the inhibition of BMPs by sensitising binding. Twisted gastrulation can also act as an independent inhibitor and levels are highest in the early stages of osteoblast differentiation (the preosteoblast stage) (Oelgeschlager *et al.*, 2003; Petryk *et al.*, 2005).

1.6.4 Gremlin

Gremlin (GREM1, locus 15q11-13; GREM2 locus 1q43), another BMP antagonist, behaves in a similar way to chordin and noggin in that it directly binds to BMP-2, BMP-4 and BMP-7, blocking their receptor interactions. It is a member of the 'differential screening-selected gene aberration in neuroblastoma'

(DAN) protein family. These proteins have an important role in skeletal function (Balemans and Van Hul, 2002). Gremlin has an effect on bone remodelling. *In vivo* studies involving transgenic mice over-expressing this protein exhibited a decreased body size, spontaneous bone fractures, modelling defects of long bones and severe osteopenia. A non-expression of the gremlin gene (known as a *homozygous null mutation*) in mice resulted in high BMP activity, which was shown to cause profound limb deformity in growing long bones. In *in vitro* studies, gremlin has been shown to inhibit BMP-induced osteoblastic differentiation (Le Blanc *et al.*, 2003; Gazzerro *et al.*, 2005).

1.6.5 Sclerostin

Sclerostin (SOST, 17q12-21) is a novel member of the DAN family that is specifically expressed by osteocytes and competes with BMP-2, -4, -6 and -7 in binding to their receptors (Kusu *et al.*, 2003). The inhibitory actions of sclerostin decrease the activity of osteoblasts, MSC proliferation and osteoprogenitor differentiation. It also has a role as a mediator of osteoblast survival and provides a significant level of regulation of bone formation by stimulating apoptosis, remodelling and repair (Sutherland *et al.*, 2004). Genetic mutations in the sclerostin gene give rise to a disorder known as sclerosteosis, which is characterised by serious lifelong bone overgrowth (Balemans and Van Hul, 2004).

1.6.6 Follistatin

Follistatin (FST 5q11.2) has a high affinity in binding BMP-7, but also binds BMP-2, -4 and -15, forming trimeric complexes (Fainsod *et al.*, 1997; Otsuka *et al.*, 2001). It has potent inhibitory mechanisms during embryogenesis and knockout mice devoid of this gene die soon after birth and display skeletal abnormalities (Matzuk *et al.*, 1995). Along with gremlin, follistatin is upregulated in osteoarthritic (OA) chondrocytes, implicating their pathologic involvement within this disease (Tardif *et al.*, 2004).

1.6.7 Anti-Inflammatory Effects of BMP-3

Bone morphogenetic protein 3 is the most abundant BMP found in adult bone and is a known antagonist of osteogenic BMPs, specifically BMP-2. It has been shown to induce osteogenic differentiation *in vitro* and to negatively regulate bone density *in vivo* (Daluiski *et al.*, 2001). Mutations in the gene for BMP-3 (BMP3 4q21.1 – 4q21.23) are associated with FOP (Schmitt *et al.*, 1999; Le Blanc *et al.*, 2003).

1.6.8 Inhibition at the Cell Receptor Level

The antagonistic mechanisms concerning inhibition at the receptor level involves targeting the signalling pathway of the members of the BMP and TGF-β superfamily, including activin. The pseudo-receptor BAMBI (BMP and activin membrane bound inhibitor) is structurally related to BMP receptors in the extracellular domain, but not the intracellular domain, preventing further signalling within the cells (Onichtchouk *et al.*, 1999; Le Blanc *et al.*, 2003).

1.6.9 Inhibition of Intracellular Processes

The following table summarises some of the proteins that are considered to be intracellular inhibitors that regulate various signalling pathways in respect of fracture healing.

Table 8: Intracellular Inhibitors

Inhibitor	Types	Actions
Inhibitory I-Smad	SMAD-6, SMAD 7	Inhibition through activation of type-I BMP receptors, reduces transcriptional activity
Smurfs (smad ubiquitin regulatory factor)	Smurf-1, Smurf-2	Inhibits BMP by degrading receptor molecules in signalling pathway
Tob	_	Anti-proliferative protein
CIZ	_	Inhibits BMP/Smad signalling pathway
Cytokines	Osteoprotegrin (OPG) Il-1 receptor antagonist (IL-1Ra)	Inhibits biological effects of specific cytokines. OPG inhibits bone resorption and induces apoptosis. IL-1Ra inhibits biological activity of IL-1.
Insulin-like growth factor binding proteins (IGFBPs)	ILGFBP-(1 – 6)	Modulates availability and stability of IGF through binding (receptor inhibition)

(Hata *et al.*, 1998; Schmidmaier *et al.*, 2000; Itoh *et al.*, 2001; Murakami *et al.*, 2003; Kwan Tat *et al.*, 2004)

1.6.10 Clinical Applications in Trauma

Statistics from the United States report that, in 1992, between 5-10% of the 6.2 million fractures resulted in either delayed healing or nonunion. Research has focused on several biological treatments for bone regeneration and fracture repair, highlighting, in particular, the usefulness of BMP on open tibial fractures (Riedel and Valentin-Opran, 1999). Indeed, the work carried out by Govender *et al.* (2002) demonstrated effective reliability of the recombinant (rh) BMP-2 in fractures, reporting a higher union rate, accelerated time to union, improved wound healing and reduced infection rate (Govender *et al.*, 2002). Other studies have found similar robustness with BMP-7 (Geesink *et al.*, 1999; Friedlaender *et al.*, 2001); both are now available as treatments for delayed healing, demonstrating the excellent osteoinductive properties of this growth factor (Dimitriou *et al.*, 2005).

1.7 THE TOXICOLOGICAL ASPECTS OF TOBACCO SMOKING

There are thought to be around 1.3 billion smokers globally and it is estimated that this results in 6 million deaths worldwide annually. Cigarette smoking has become the single most avoidable cause of morbidity and mortality and it is expected that 1 billion people will die as a result of smoking in the 21st century (Wipfli and Samet, 2009). In the United Kingdom in 2004, 25% of adults smoked, according to the Office for National Statistics (Gruer *et al.*, 2009). Orthopaedic surgeons have long known about the complications of smoking that can harmfully influence the dynamics of bone healing (Porter and Hanley, 2001). The damaging effects of tobacco smoke are dose-related and as such are perhaps reversible by cessation. However, the habit is very addictive and many people who take up smoking before they reach twenty years of age continue to smoke for many years, often up until death (Bartecchi *et al.*, 1994). The musculoskeletal effects may become more apparent as treatments for smoking-related conditions become more effective and individuals survive longer (Capewell *et al.*, 2009).

The adverse effect of smoking on healing has been the basis of numerous clinical studies since Mosely and Finseth found that cigarette smoking had a deleterious effect on the healing of hand wounds in 1977 and there is a strong association with cardiovascular and respiratory diseases (Mosely and Finseth, 1977; Rees *et al.*, 1984; Riefkohl *et al.*, 1986; Kroll, 1994). Research findings have suggested that nicotine and related chemicals in cigarette smoke can also impair the regeneration of healing wounds and soft tissue in limbs following fracture injury, thereby decreasing the quality of postoperative outcomes and prolonging healing times (Nolan *et al.*, 1985; Netscher and Clamon, 1994; Pitts *et al.*, 1999).

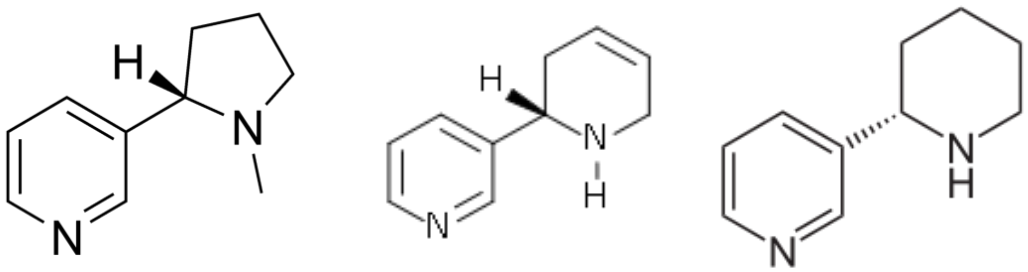
1.7.1 The Constituents of Cigarette Smoke

Two types of cigarette smoke exist, 'mainstream' smoke and 'sidestream' smoke. The former is inhaled by the smoker from the filter-tip end of the cigarette; the latter is the smoke that is released into the local environment from the burning end of the cigarette. The mainstream smoke is rich in reactive oxygen species (ROS) and reactive nitrogen species (RNS), termed 'free radicals', both of which can cause cellular damage (and/or death) via oxidative stress processes (Pryor, 1997; Comhair and Erzurum, 2002). Approximately 2-3 mg of nicotine and 20-30 mL of carbon monoxide are inhaled from each cigarette, depending on the brand and tar content (Sherwin and Gastwirth, 1990). The toxins that are implicated in the cardiovascular morbidity found in cigarette smokers are also responsible for the damage inflicted on the musculoskeletal system (Toyooka and Ibuki, 2009).

Both types of cigarette smoke consist of volatile acids, known as the *volatile phase* and also complex compounds of particulate matter, known as the *particulate phase*. The volatile phase is larger and accounts for 95% of the cigarette smoke; in this phase, approximately 500 different gases are released, including nitrogen (N_2), carbon monoxide (CO), carbon dioxide (CO_2), ammonia (NH_3), hydrogen cyanide (HCN) and benzene (C_6H_6). Around 3500 chemicals are produced during the particulate phase and include nicotine ($C_{10}H_{14}N_2$), anatabine ($C_{10}H_{12}N_2$) and anabasine ($C_{10}H_{14}N_2$) (Figure 17) (Hoffmann and Hoffmann, 1997). The tar, which is produced when the particulate matter loses water, contains the carcinogens in cigarette smoke (Zevin *et al.*, 1998). Hydrogen cyanide is a noxious gas that is found in the bloodstream of smokers; its primary

effect is at the cellular level, disrupting enzymatic oxidative metabolism (Whiteford, 2003).

Figure 17: The Chemical Structures of Nicotine, Anatabine and Anabasine



Nicotine ($C_{10}H_{14}N_2$) **Anatabine ($C_{10}H_{12}N_2$)** **Anabasine ($C_{10}H_{14}N_2$)**

(Hukkanen *et al.*, 2005)

1.7.2 Nicotine

Regarded as the addictive component in tobacco, nicotine has been shown to increase platelet aggregation, decrease microvascular prostacyclin levels and inhibit the biological function of fibroblasts, red blood cells and macrophages. It is a toxic alkaloid that becomes bound to nicotinic acetylcholine (ACh) receptors. The mechanism of action is complex and the drug serves as both a ganglionic depressant and stimulant; catecholamine levels in the bloodstream become increased, promoting the formation of chalones as the cigarette is smoked. This process stimulates cardiac output and causes adrenergic vasoconstriction. The chalones also inhibit epithelialisation, undermining the healing process (Lefkowitz, 1976; Waeber *et al.*, 1984; Winniford *et al.*, 1986; Cobb *et al.*, 1994; Al-Hadithy *et al.*, 2012).

Nicotine decreases blood flow to the extremities due to the increased peripheral vasoconstriction, especially relating to digital and forearm haemodynamics (Bornmyr and Svensson, 1991). Indeed, smoking just 2 cigarettes was found to decrease blood flow to the hand by 29 % (van Adrichem *et al.*, 1992). Nicotine also influences blood coagulation by increasing platelet aggregation, causing the sludging of blood in the small vessels and a generalised reduction in microvascular perfusion (Mosely and Finseth, 1977). Blood viscosity and fibrinogen levels are both raised, inducing a state of hypercoagulation, thus, increasing the risk of polycythaemia and microvascular clotting (Folsom, 1995; El-Zayadi *et al.*, 2002; Balaji, 2008).

Nicotine has a significant and wide ranging effect on plasma hormone levels; vasopressin, B-endorphin, adrenocorticotropic hormone (ACTH) and cortisol are all raised in the circulation of smokers (Seyler *et al.*, 1984; Seyler *et al.*, 1986). At the cellular level, nicotine damages osteoblasts, fibroblasts and macrophages (de Vernejoul *et al.*, 1983; Nolan *et al.*, 1985; Sherwin and Gastwirth, 1990; Fang *et al.*, 1991). There is an increased risk of atherosclerosis due to the damage caused to the vascular endothelium by the toxins inhaled with smoking (Krupski, 1991; Balaji, 2008).

1.7.3 Carbon Monoxide

Carbon monoxide has a 200-fold greater affinity for haemoglobin binding than oxygen, which reduces O_2 tension in tissues (Jorgensen *et al.*, 1998; Leow and Maibach, 1998). The toxic gas arises as a consequence of incomplete paper and

tobacco combustion, forming carboxyhaemoglobin as it binds preferentially with the haemoglobin molecule. Cigarette smoke is known to contain 2-6% CO and can convert up to 15% of haemoglobin into carboxyhaemoglobin. The formation of carboxyhaemoglobin (as opposed to oxyhaemoglobin) attenuates O_2 transport and dissociation, resulting in hypoxia (Rees *et al.*, 1984; Nolan *et al.*, 1985; Barnoya and Glantz, 2005). Ten minutes of smoking has been shown to reduce tissue O_2 tension in tissues for 1 hour and a regular smoker consuming 20 cigarettes per day, therefore, is tissue-hypoxic for 20 hours each day (Sorensen *et al.*, 2009).

1.7.4 Cigarette Smoking and Cellular Damage

Mesenschymal stem cells, fibroblasts, acute-phase proteins and growth factors are crucial mediators of fracture repair and become exposed to the circulating components that are present in the bloodstream associated with cigarette smoking (Czernin and Waldherr, 2003). The adverse effects can be seen in the skin of chronic smokers who develop a loss of dermal elasticity, which arises because of abnormal fibroblast function (Frances *et al.*, 1991). Patients who smoke are advised by their surgeon not to do so perioperatively, not only to improve lung and cardiovascular function, but also to optimise the healing process (Akoz *et al.*, 2002). The morbidity associated with tobacco smoke inhalation presents challenges in terms of defective healing and also over-healing, which are not limited to merely cosmetic problems; a systemic underlying impediment is believed to exist (Siana *et al.*, 1989; Sorensen *et al.*, 2002; Sloan *et al.*, 2010).

Mesenchymal stem cells and fibroblasts are key producers of cytokines that are instrumental in the induction of the initial inflammatory response and have an important role in the formation of granulation tissue in bone healing. Chemokines, for example, which are vital in attracting neutrophils and other leukocytes to the wound area, are secreted by MSCs and fibroblasts (Wong and Martins-Green, 2004).

Successful fracture repair is highly dependent on tightly-controlled mechanisms that are directly involved in the expansion of fibroblast-rich granulation tissue. Severe consequences can arise if this process is deregulated and an excessive fibrotic reaction and impaired healing can ensue. Cigarette smoking is thought to influence and exacerbate these pathobiological processes (Gabbiani, 2003; Wong and Martins-Green, 2004; Patel *et al.*, 2013) .

Reactive oxygen species produced by smoking have an effect on mitochondrial morphology, but the specific mechanisms influencing this are not clear. The best studied catalyst for mitochondrial fission, however, implicated the dynamin-like protein-1, which has been shown to cause aberrant mitochondrial morphology, or 'punctuated' mitochondria, under environmental stress (Smirnova *et al.*, 1998; Pitts *et al.*, 1999; Frank *et al.*, 2001).

The precise biological effects of cigarette smoke on cells remain largely unknown due to a lack of scientific research in this area. However, Wong and Martins-Green (2004) found that non-lethal levels of mainstream whole smoke (MSW) led to the inhibition of fibroblast migration, which is a vital aspect of the healing

process. This study concentrated on fibroblast cells explanted and cultured from chicken embryos and it was discovered that even moderate levels of nicotine, whilst not in sufficient quantities to kill cells, did have a serious effect on them. Indeed, modest doses of MSW can affect ATP production by primary fibroblasts, stimulating the production and activation of stress response proteins that are recognised as *survival factors*. Survival factors increase cell lifespan, inhibiting apoptosis. They have been shown to result in the accumulation of fibroblasts and consequently a build-up of connective tissue, leading to excessive and detrimental healing, fibrosis and scarring of tissues (Wong and Martins-Green, 2004). Sustained stimulation of survival factors has implications for diseases such as cancer and fibrosis and levels of the p53 and p21 protein become increased, allowing cells to repair their DNA during cell cycle arrest (Helt *et al.*, 2001; Whyte *et al.*, 2002; Patel *et al.*, 2013).

Cigarette smoke disrupts the microtubule arrangement in fibroblasts that can disrupt effective healing. Microtubules are major cytoskeletal elements that are involved in the transportation of signalling molecules and organelles to different parts of the cell, enabling them to carry out their tasks effectively. The effect of smoking on microtubule organisation impinges on mitochondrial function, since mitochondria are located along these microtubules, as opposed to being randomly distributed throughout the cell. Therefore, changes in microtubule structure are likely to affect the distribution and morphology of these organelles. A disordered microtubule function can also contribute to mitochondrial fragmentation and accumulation in the perinuclear region. This can inevitably give rise to a reduced amount of ATP production (Hollenbeck, 1996; Rinker, 2013).

A principal function of fibroblasts in the healing process is migration into the wound area in order to secrete growth factors and cytokines and to deposit and remodel the ECM. A disruption to this process will give rise to poor healing and can lead to chronically unhealed wounds in the patient and is a common problem associated with smoking (Wong and Martins-Green, 2004).

1.8 CIGARETTE SMOKING AND FRACTURE HEALING

Over one million fractures are sustained each year in the United Kingdom (3.6 per 100 people per year) and 5% to 10% are reported not to heal satisfactorily. The prevalence of lifetime fracture is over 50% in middle-age men and 40% in women aged over 75. Fracture is less common in non-white population and this is seen in the majority of black and minority ethnic groups, as reported in the literature (Donaldson *et al.*, 2008). It is imperative that the orthopaedic surgeon is aware of the likely factors that are associated with impaired healing of bone, so that they may be avoided whenever practicable. There are numerous theories as to how smoking can impact on the fracture healing process and include a reduced blood supply to the injury site, high levels of reactive oxygen intermediates in the circulation, low levels of antioxidants and vitamins and the attenuating effect nicotine has on endothelial nitric oxide synthase (eNOS). There are also findings that high doses of nicotine, associated with cigarette smoking, are toxic to proliferating osteoblasts and calcitonin (Gaston and Simpson, 2007; Lee *et al.*, 2013). The role of the hormone calcitonin opposes that of PTH and is crucial in regulating levels of circulatory Ca^{2+} in the blood. Smoking is said to increase levels of calcitonin, which in turn lowers calcium levels, making it less available

for bone formation in fracture healing (d'Herbomez *et al.*, 2007). Bone health is compromised by cigarette smoke and is well known to contribute to accelerated osteoporosis and femoral osteonecrosis in men as well as women (Wright, 2006). However, female smokers were found to commence menopause two years earlier than non-smoking women, presenting a greater risk for osteoporosis (Baron, 1984). Fracture rates in smokers tend to be higher due to increased skeletal fragility and higher rates of accidents; work-related injuries and automobile collisions are also more common in smokers. The explanation as to why smokers are more at risk of injury is unclear, although general weakness, inferior balance and poorer neuromuscular function have been identified as possible reasons (Kyro *et al.*, 1993; Nelson *et al.*, 1994; Sacks and Nelson, 1994; Porter and Hanley, 2001).

The adverse effects of smoking on fracture healing were reported by Whitesides *et al.* (1994) in patients that underwent spinal fusion (spondylosyndesis or arthrodesis), with smokers generally having a higher rate of pseudoarthritis than the non-smoking control groups (40% vs. 8%), which was supported in another study that showed smokers who underwent instrumental spinal infusion suffered increased rates of nonunion compared to non-smokers (26.5% vs. 14.2% ($p<0.05$)) (Glassman *et al.*, 2000). Several clinical studies have been carried out on human subjects regarding fracture healing and smoking (Whitesides *et al.*, 1994; Silcox *et al.*, 1995). Carpenter *et al.* (1996), reported that outcomes of lumbar fusion operations were significantly more favourable for non-smokers ($p<0.02$). The group also found that non-smokers were more likely to return to work on time than smokers following this surgical procedure (Carpenter *et al.*, 1996).

A seminal trial undertaken by Adams *et al.* (2001), involving 140 smoking and 133 non-smokers, found that the mean time to union of open tibial fractures was 32 weeks for smokers, compared with just 28 weeks for non-smokers. The authors also found that, in the same groups of patients, bone grafting was necessary in 36 smokers, but only 24 non-smokers required the procedure in order to stimulate bone healing (Adams *et al.*, 2001). These results were statistically significant ($p<0.05$) and showed that smoking is deleterious to human fracture healing. The findings were consistent with previous experimental studies, which also demonstrated a nicotine-impaired blood flow to the fracture site (Nolan *et al.*, 1985; van Adrichem *et al.*, 1992).

High doses of nicotine were reported to have a toxic effect on proliferating osteoblasts but, in smaller doses, it actually stimulated the growth of these cells (Gullihorn *et al.*, 2005). This paradoxical function was supported by another study, where it was shown that tobacco extract not containing nicotine impaired the mechanical strength of femoral fractures in rats, whereas nicotine alone had no detrimental effect on the healing of the animals. In fact, nicotine was later found to actually increase the strength (perhaps due to over-healing) of repaired bones in a dose-dependent manner in rat femurs (Skott *et al.*, 2006). These results suggest a possible benefit of low dose nicotine replacement therapy (NRT) in smokers who, after sustaining a fracture, have been instructed to cease smoking in the peri- and postoperative recovery period. The data suggests that it is the other components in the smoke that can posses osteoblast damaging properties (Gaston and Simpson, 2007). However, nicotine was actually shown to inhibit repair in a different fracture model (Raikin *et al.*, 1998).

Surgical fixation is recommended for fractures that are not able to be reduced by external, non-surgical methods. This is especially true of fractures that involve joints, as iatrogenic mal-alignment of articular surfaces may contribute to the development of arthritis and nonunion. Once the patient has been anaesthetised, the bone is placed in the correct position and screws, pins, or plates are attached to or placed in the bone either temporarily or permanently. Alternatively, long bones may be fixed with nails which are normally placed in the medullary cavity to support immobilisation (Shapiro, 2008).

The cessation of smoking, therefore, is routinely advised perioperatively, but temporal guidelines are vague as conclusive studies regarding this have yet to be undertaken. The few published recommendations state that smoking cessation should commence 1-21 days preoperatively (where permissible) and 5-28 days postoperatively (Campanile *et al.*, 1998; Rinker, 2013). Twelve hours prior to surgery is certainly a minimum, as this is the amount of time required to clear the human body of CO (Sherwin and Gastwirth, 1990). Five smoke-free days prior to surgery has been documented as having a favourable outcome on wound healing (Abidi *et al.*, 1998), although one week was implicated by Lind *et al.* (1991) due to the pharmacokinetics of free radicals and thrombotic components (Lind *et al.*, 1991).

However, longer periods of abstinence have been suggested than those recommended by Campanile *et al.* (1998) and Lind *et al.* (1991). Whitesides *et al.* (1994) showed that non-smokers took 2 months to produce 1.0 cm of bone, whereas smokers took 3 months to grow the same amount of tissue. This led to

the suggestion by the authors that a period of 60 smoke-free days should elapse before surgery can take place in elective spinal arthrodesis patients (Whitesides *et al.*, 1994). On the other hand, Porter and Hanley (2001) have argued that smoking causes irreversible damage to tissues and, therefore, surgical treatment should not be denied on the grounds of the failure to stop smoking until further research has been carried out in this area (Porter and Hanley, 2001).

1.9 SUMMARY OF INTRODUCTION

Fracture regeneration is a complex process involving many factors and molecules that are present at and recruited to, the site of injury during the inflammatory, proliferative and maturation phases of healing. The effects of smoking are thought to be mediated via the vasoconstrictive, platelet-activating and aggregating properties of nicotine, the hypoxia-promoting effects of carbon monoxide and the inhibition of oxidative metabolism by hydrogen cyanide at the cellular level.

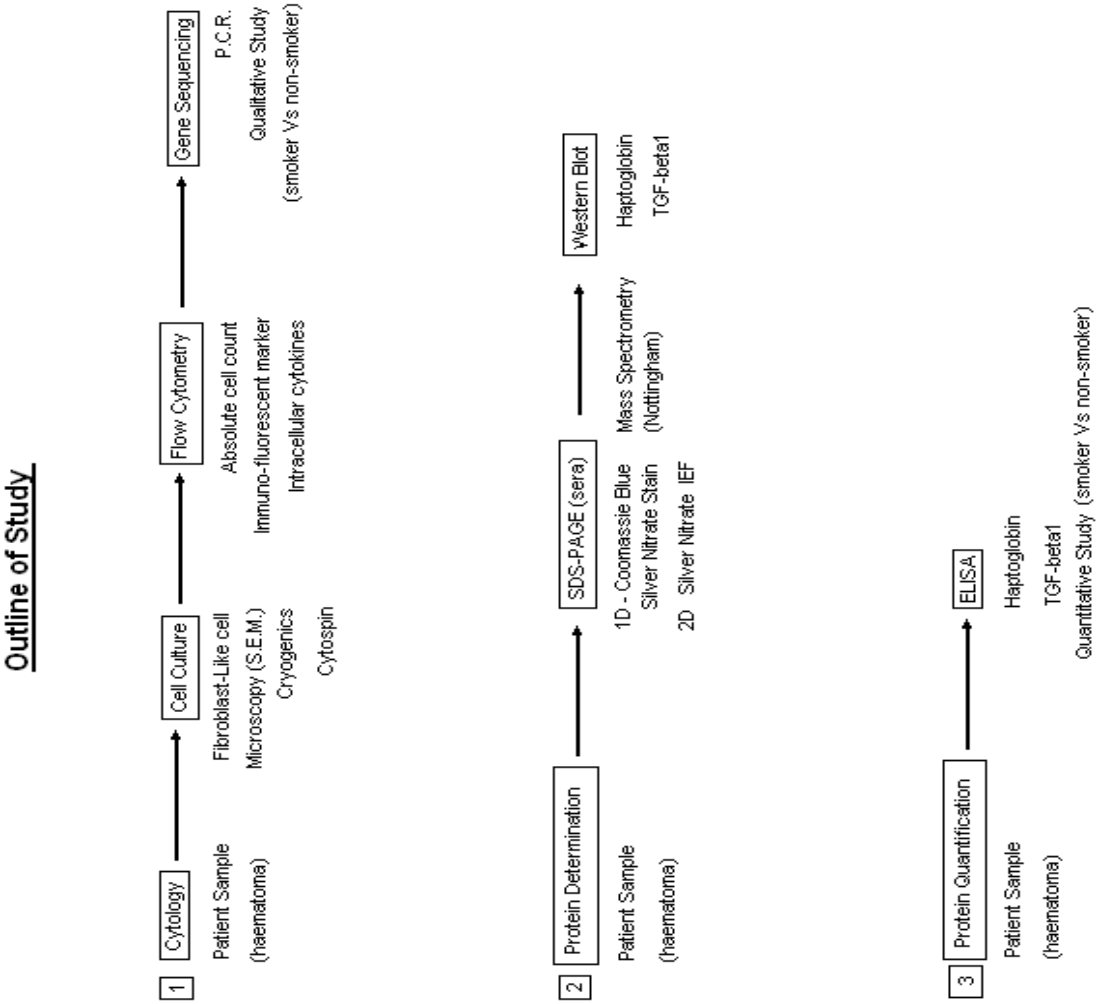
The mechanisms that take place in response to the trauma are temporal in nature and in the majority of cases, follow a specific pathway that is tightly controlled and regulated by many mediators of both stimulatory and inhibitory origin. Tobacco smoking is known to have a deleterious effect on the dynamics of both wound and fracture healing and numerous studies involving animals and humans have been undertaken to demonstrate this. However, very few investigations at the cellular and molecular level involving human fractures and smoking have been carried out. Orthopaedic surgeons have recommended that there should be a

period of smoking cessation prior to surgery and therefore a history of cigarette smoking should be obtained from individuals so that the risks and complications may be discussed in detail. Both surgeon and patient should be completely aware of the harmful effects of smoking when assessing or planning a surgical intervention. These observations notwithstanding the evidence available to date, strongly implicate tobacco smoking in inducing delayed and nonunion of bone during fracture healing and ultimately suggests that smoking should be avoided by patients who attend hospital with a fracture.

1.10 *Aims and Objectives*

There are two aims to this study. The ***first aim*** is to isolate and culture MSCs from fracture haematomas collected from those eligible and consenting patients who have sustained a tibial fracture. The donated tissue is to be processed within the first 24 hours of injury. The extracted MSCs are then analysed using various methods, in order to observe the biochemical differences and to make comparisons between those cells from the smoking patients and those from non-smoking patients. The inclusion of a smoking model can also be used to assess some of the impacts of smoking on cells extracted from the fracture haematoma. The ***second aim*** is to compare the expression of acute phase proteins that are involved in the early healing phases of fracture repair. In order to accomplish this, extra- and intracellular cytokines will be analysed in both the sera extracted from the fracture haematoma and cell cultures. A variety of techniques will be employed to separate, determine and quantitate acute-phase proteins of interest and a summary outlining these methods can be found in Figure 18, overleaf.

Figure 18: Proposal for Outline of Study



2 CHAPTER 2

MATERIALS AND METHODS

2.1 METHODS I: CELLULAR ASPECTS

2.2 SPECIMEN PROCESSING

2.2.1 *Patient Handling*

Those patients taking anticoagulants, steroids or anti-inflammatory drugs in the three months prior to injury were excluded from the trial. The study involved adult patients from each group, as detailed below in *Table 9.* The age and sex of each patient, ethnic group and daily number of cigarettes smoked (where applicable) was noted and logged and this paperwork accompanied each sample. Informed written consent from all patients, as well as ethical approval from the NHS Research Ethics Committee (application ref: 06/Q2401/80) and the University of Lincoln Ethics Committee (GS4) was obtained.

Table 9: Sample Information

	Total Respondents (Smokers vs. Non-smokers)	**Smokers (Cells)**	**Non-Smokers (Cells)**	**Smokers (Serum)**	**Non-Smokers (Serum)**
Male	34 (11 vs. 20)	5	8	10	15
Female	14 (4 vs. 10)	2	3	4	10
Total	48 (18 vs. 30)	7	11	14	25

Table 9 above shows the outcome of respondents' fracture haematomas in terms of tissue type. Of the 48 fracture patients recruited into the study, 18 specimens produced progenitor cells (7 smokers vs. 11 non smokers). Serum was derived from 39 patient samples (14 smokers vs. 25 non-smokers). Male casualties were

more common than females, representing a male-to-female ratio of more than 2:1. Medical notes and x-ray radiographs of patients' fractures were reviewed periodically to identify any clinical problems or comorbidities with regard to the healing process.

Health and Safety: The Control of Substances Hazardous to Health (CoSHH) Regulations 2002 have been strictly adhered to and appropriate risk assessments were in place throughout the entirety of all of the following experiments. Designated laboratory coats were worn at al times, along with nitrile examination gloves and eye protection. Enhanced personal protective equipment was used when handling liquid nitrogen (N_2) due to an increased risk of freeze-burn.

2.2.2 Extraction and Isolation of Haematoma Cells (HCs)

The following basic materials and consumables are required for the haematoma cell culturing experiments:

Product	**Amount Required**
SIGMA-ALDRICH (Gillingham)	
Phosphate Buffered Saline (PBS)	1 L
Iscove's Modified Dulbecco's Medium (IMDM) (17633)	1 L
Trypsin EDTA (T3924)	500 mL
Foetal Calf Serum (FCS) (N4762)	500mL
Penicillin/Streptomycin (P4333)	100mL
Dimethylsulfoxide (DMSO) (D2659)	x5 vials
L-Glutamine (200mM)	200mL

(cont.)

Product	**Amount Required**
SARSTEDT (Nümbrecht)	
25 cm2 Cell Culture Flask (coated)	500
Pipetboy Tubes:	
25 mL	500
5 mL	500
Cryotube (RY960257)	500
Blue pipette tips	

2.2.3 Tissue Collection and Processing

1. After exposure of the fracture site, the haematoma (~2.0 mL) was recovered manually and placed in an untreated sterile polypropylene container (Fisher, Loughborough) by theatre staff at Lincoln County Hospital. Patient consent forms were obtained, completed and placed on file.

2. The sample was immediately transported to the University of Lincoln Biomedical Research Laboratory, on wet ice, for processing.

3. A primary culture was prepared by placing ~8 small droplets of the semi-solid haematoma onto the base of a 25 cm^2 non-coated culture flask (Sarstedt, Nümbrecht).

4. The spots of haematoma (see step 3) were allowed to clot for ~1 hour.

5. 50.0 mL of Iscove's Modified Dulbecco's Media (IMDM) containing 10% Fetal Calf Serum (FCS), 100 μg mL^{-1} Streptomycin, 100 U mL^{-1} Penicillin antibiotic, 500 μL of Fungizone antifungal reagent (equivalent to 0.25 μg mL^{-1} amphotericin B) and 2.0 mM L-glutamine (all Sigma-Aldrich, Gillingham), was prepared as the stock cell-culture medium.

6. Using a disposable Pasteur pipette, 5.0 mL of the stock cell culture solution (preheated to 37ºC) was carefully dispensed into each of the cell culture flasks (Sarstedt, Nümbrecht) so as not to disturb the clots of blood. Each flask and its

contents were then placed in a Galaxy R incubator (Wolf Laboratories, York), which was set at 37°C with humidified 5% CO_2. Ambient O_2 levels (approximately 20-21%) are reported by the manufacturer to be standard with this model of incubator.

7. After 7 days of incubation, 2.5 mL of the cell culture supernatant was removed from the flask and safely discarded into a solution of Virkon disinfectant.

8. 2.5 mL of freshly-prepared culture media (as step 5) was dispensed into each flask, which were then returned to the incubator (as step 6). The media was changed on a regular basis (every 4-5 days).

9. Serum was also extracted from the remainder of the specimen for analysis; this was achieved via centrifugation (30 mins; 3500 rpm; 4ºC) (Beckman Coulter Allegra X-12R, California) in a collection tube manufactured with a gel serum separator (Vacuette, Kremsmünster).

2.2.4 Isolation of Haematoma Cells (HCs) via Subculture

1. After ~21 days of primary-culture, the adherent cells were harvested from the primary culture flasks by passing 2.0 mL proteolytic enzyme solution of 0.25% trypsin, containing 0.02% ethylenediaminetetra-acetic acid (EDTA) (Sigma-Aldrich), onto the haematoma cell (HC) surface monolayer. The flasks were placed in the incubator (as previously described) for 5 minutes.

2. The solution, containing, media, trypsin and cells, was withdrawn from the vessel and dispensed into a centrifuge tube using a serological pipette. The flask was then washed out with 2.0 mL of phosphate buffered saline (PBS), which was also added to the same centrifuge tube.

3. 2.0 mL of the complete IMDM was immediately added to the solution of HCs, trypsin and EDTA so that the proteolytic enzyme became neutralised.

4. The solution was centrifuged at 1200 rpm, 18°C for 15 minutes (Beckman Coulter Allegra X-12R, California). A cell-pellet formed at the base of the tube; the resulting supernatant was safely discarded by pouring swiftly into a receptacle.

5. After *tapping-out* the pellet to disperse cells, 1.0 mL of the prepared complete media solution (from step 3) was added to form a suspension of HCs. The cell suspension was dispensed into a non-coated cell-culture flask (Sarstedt, Nümbrecht) that contained 5.0 mL of fresh complete media.

6. The flasks were labelled *1st passage* and then placed in the incubator as previously; the media was changed every 5-7 days thereafter. Figure 19 (overleaf) illustrates the processes involved in cell culture.

7. A control culture was produced, employing the stated methods above, using a commercially available human mesenchymal stem cell line (Lonza, Tokyo).

Figure 19: Schematic Diagram of Methods Involved in Extraction and Isolation of Haematoma Cells (HCs)

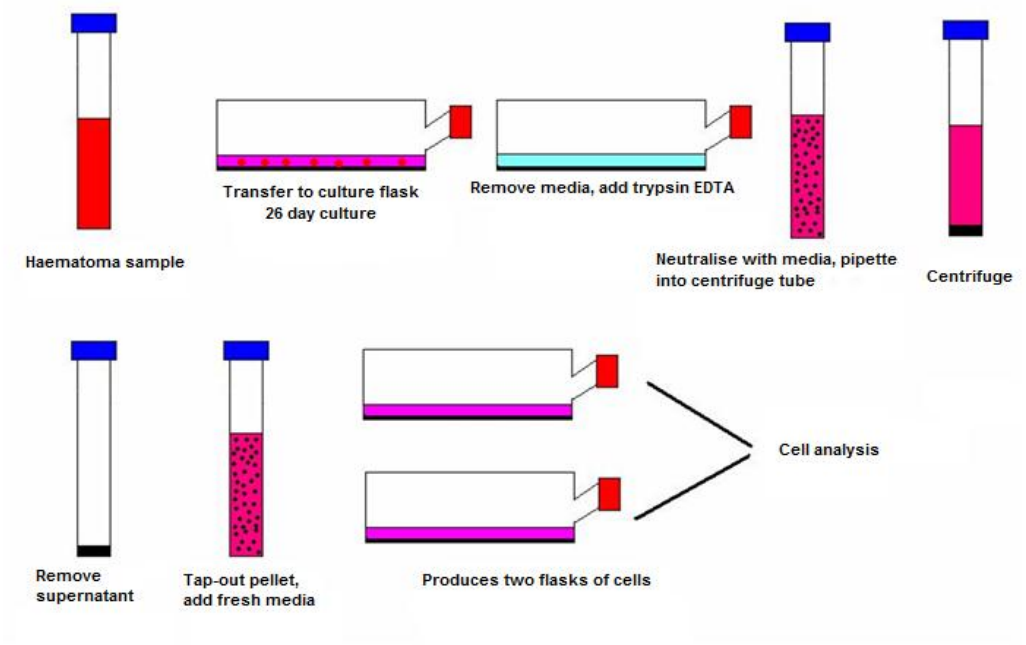
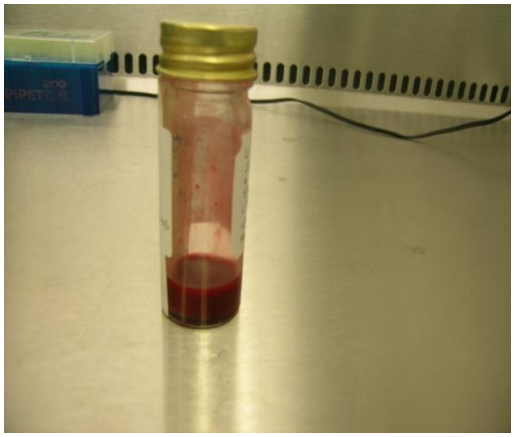
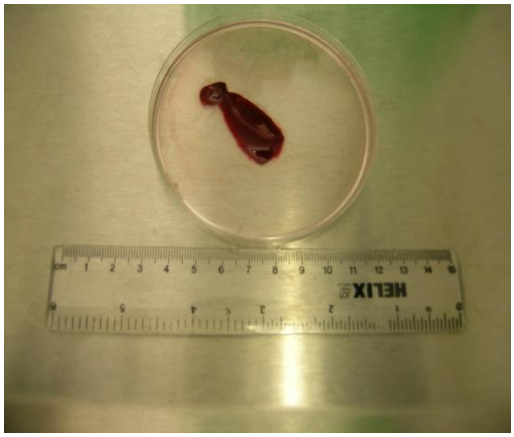


Figure 19: The stages of processing the patient sample. The excised haematoma tissue is placed into a sterile polypropylene sample tube in theatre. The clotted portion is removed and washed in PBS prior to explanting into tissue culture flasks in small pieces (~2.0 mm^3). After allowing to clot for approximately 1h, 5.0 mL of complete culture media is added before incubation for approximately 26 d.

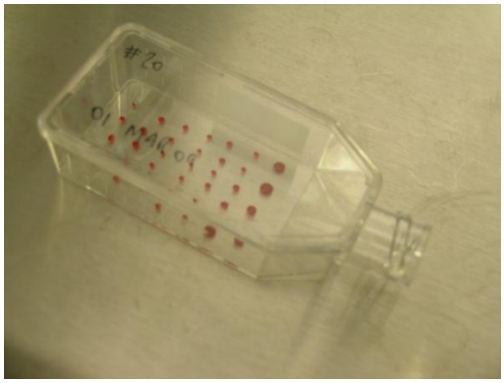
Figure 20: Fracture Haematoma Processing



a) The Excised Haematoma from theatre



b) Sample Washed in PBS



a) The Explanted Haematoma

d) Addition of Culture Media

Figures 20 (a-d): The stages of haematoma processing from a) receiving the excised sample from theatre before b) washing in PBS, c) explanting the specimen into tissue culture flasks and allowing solidification before d) finally adding complete IMDM complete culture media.

2.2.5 Cell Counting Using Flow Cytometry

1. Flow cytometric absolute cell counting was used to generate data for the purpose of cell-seeding and cell-doubling analysis. It was also used to assess cellular recovery in later experiments.

2. The Epics XL flow cytometer (Beckman Coulter, High Wycombe) was switched on and the system was allowed to become pressurised (~15 minutes), after which a 'flowcheck' was carried-out (as per manufacturer's standard operating procedures). This ensured that the item of equipment was calibrated correctly. (See appendices for flowcheck documentation).

3. 100 μL of the 1000 μL cell suspension (following subculture protocol) was combined with 1000 μL of Isoton 3 solution and 100 μL of fluorescent flow-count beads (Beckman Coulter, California), producing a final solution of 1200 μL.

4. 1200 μL of the solution (see step 3) was transferred to a cytometer tube by means of an automatic pipette. The tube was inserted into the stage of the flow cytometer.

5. A suitable protocol programme, or listmode (LMD), was selected from the software before running the sample. After the processing the specimen, data was collected and the machine was cleansed by running a standard common cleaning panel protocol.

2.3 CHARACTERISATION OF HAEMATOMA CELLS

In addition to being counted, cells were labelled with antibodies specific to mesenchymal lineage and the markers CD29 (Caltag, Carlsbad, CA; Cat#LCD2901), CD44 (Caltag, Carlsbad, CA; Cat#MHCD4401), CD73 (phycoerythrin conjugate (PE)) (eBioscience, San Diego; Cat# 12-0731-82 Lot#E027728), CD105 (Immunostep, Salamanca; Cat#105F Batch# 180608/0105) and CD166 (Medical and Biological Laboratories Co., Naka-ku Nagoya Cat#K0044-4 Lot011) were used in both the immunofluoresence (not CD73 as the conjugate was phychoerythrin) and immunophenotyping assays. The haematopoietic stem cell marker CD34 (Novocastra Laborotories Ltd, Newcastle upon Tyne; Cat#QBEnd/10) was used as a negative control, in addition to staining an un-probed sample of cells with secondary antibody only (rabbit, anti-mouse) (DAKO Cytomation, Ely Cat#PO260 Lot 00043039). The protocols used for the assays are as follows:

2.3.1 Immunofluorescence Assay

1. After the application of the trypsin EDTA and the re-suspension into 1.0 ml of complete media, the cell suspensions were placed in a cytospin (Thermo Shandon, Runcorn) in order that they became uniformly fixed onto a glass microscope slide. 200 μL of the cell suspension containing approximately 5 x 10^4 was pipetted into a cytofunnel chamber that was attached to a filter and clamped to a glass slide (Thermo Shandon, Runcorn).

2. The apparatus was programmed to run at 700 rpm for 5 minutes. After completion of the cycle, cytofunnels were carefully removed to reveal a 'button' of cells that had formed on the glass slides. Cells were fixed with ice-cold pure methanol and air-dried; slides were wrapped in aluminium foil and then placed in a freezer for storage (-20ºC).

3. After removing from the freezer, cells were briefly thawed at room temperature (~1 minute). 50 μL of human monoclonal primary antibody (mouse; anti-human, conjugated to fluorescein isothiocyanate (FITC); CD29, CD44, CD105, CD166 or CD34) was pipetted onto the cell button and left to incubate at humidified room temperature for 30 minutes. One slide was stained with FITC secondary antibody only as an additional control.

4. Cells were gently washed using phosphate buffered saline (PBS). 50 μL diluted (1:30) secondary polyclonal antibody (rabbit; anti-mouse) (Sigma-Aldrich, Gillingham) was pipetted onto the cells, which were then incubated in the dark at humidified room temperature. This was necessary to further amplify the signal from the already-conjugated antibody so that it could be seen under fluorescent microscopy.

5. Following incubation, cells were washed once again in PBS and glass cover slips were applied to the slides. Samples were analysed immediately using a fluorescent microscope (Nikon, Livingston), which was set to the FITC mode.

2.3.2 Immunophenotyping Assay

1. After being subjected to trypsin/EDTA, centrifugation and re-suspension, harvested cells were counted using a flow cytometer and specific amounts (5 x 10^5) were each diluted in 900 μL of Isoton II diluent (Beckman Coulter, High Wycombe).

2. 10 μL of monoclonal primary antibody (either CD29, CD34, CD44, CD105 or CD106), conjugated with fluorescein isothiocyanate (FITC), was also added to the solution and left to incubate at room temperature in the dark for 30 minutes.

3. Samples were then processed on a flow cytometer to quantify the level of positive cells within the cell population for each given CD marker, shown as normalised percentages.

2.3.3 Generation of Cigarette Smoke Extract (CSE)

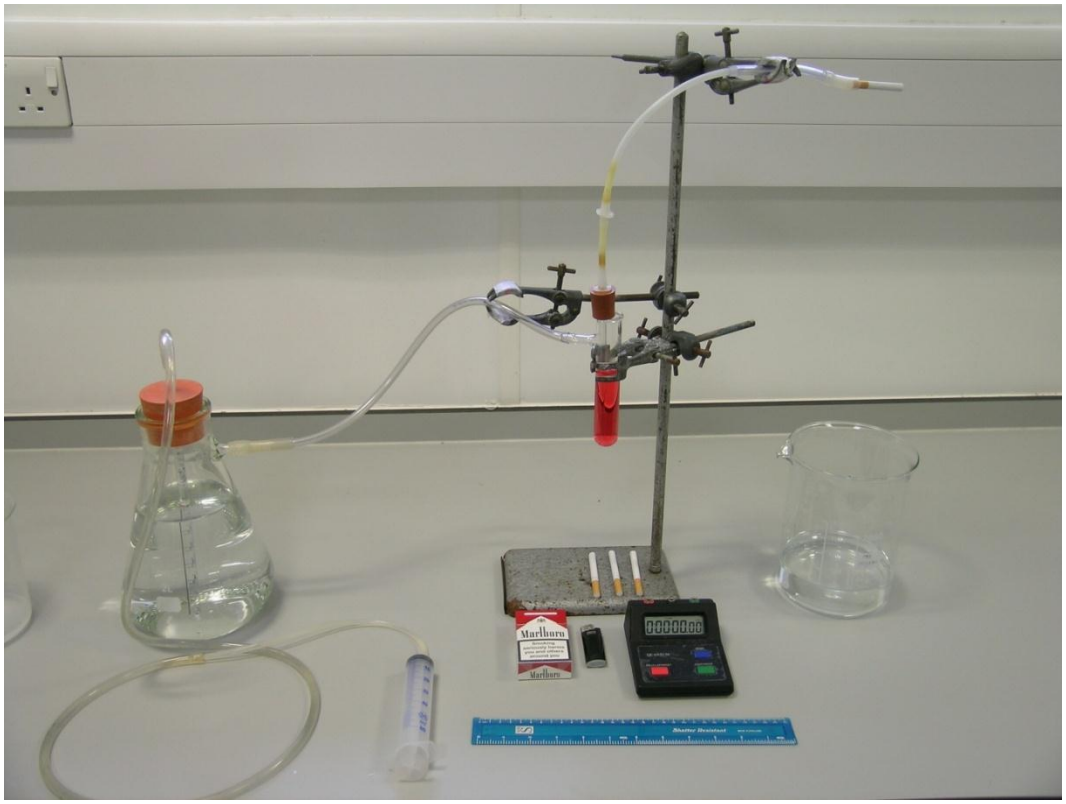


Figure 21: Apparatus set-up for the generation of Cigarette Smoke Extract (CSE)

The cigarette smoke extract (CSE) generation was based on a validated pump system as reported by Bernhard *et al.* (Bernhard *et al.*, 2004). Four cigarettes were smoked through 30 mL of IMDM. Each cigarette was smoked for 10 puffs, each puff being 35 mL of smoke every 30 seconds, which burnt approximately 75% of the cigarette. The volume of smoke generated was therefore 350 mL. Each millilitre of CSE contains 0.133 (4/30) of a cigarette's smoke-derived constituents. The resultant CSE was sterilised by filtering through a 0.2 μm

syringe filter and the pH was adjusted to 7.4, pipetted into 1.0 mL aliquots and frozen at -20ºC. The brand of cigarettes used was Marlboro Reds Class A and were rated as 10.0 mg tar, 0.8 mg of nicotine and 10.0 mg of carbon monoxide.

Bernhard's validated volumetric equations are based on the assumptions that a human generates 350 mL of smoke with each cigarette and has a blood volume of 6L. A person smoking 20 cigarettes per day will have the equivalent of 1 cigarette's toxins in each 300 mL (6000/20) of blood. Therefore, each mL of blood contains 0.0033 (1/300) of 1 cigarette's smoke-derived constituents. By this calculation, CSE can be considered to contain 40 times (0.133/0.0033) the amount of smoke-derived constituents that would be expected to be contained in the blood of a smoker who smokes 20 cigarettes per day. A 2.5% (1/40) solution of CSE in the IMDM would, therefore, equate to 20 cigarettes per day.

2.3.4 Nitric Oxide (NO) Analysis in CSE-Treated MSCs

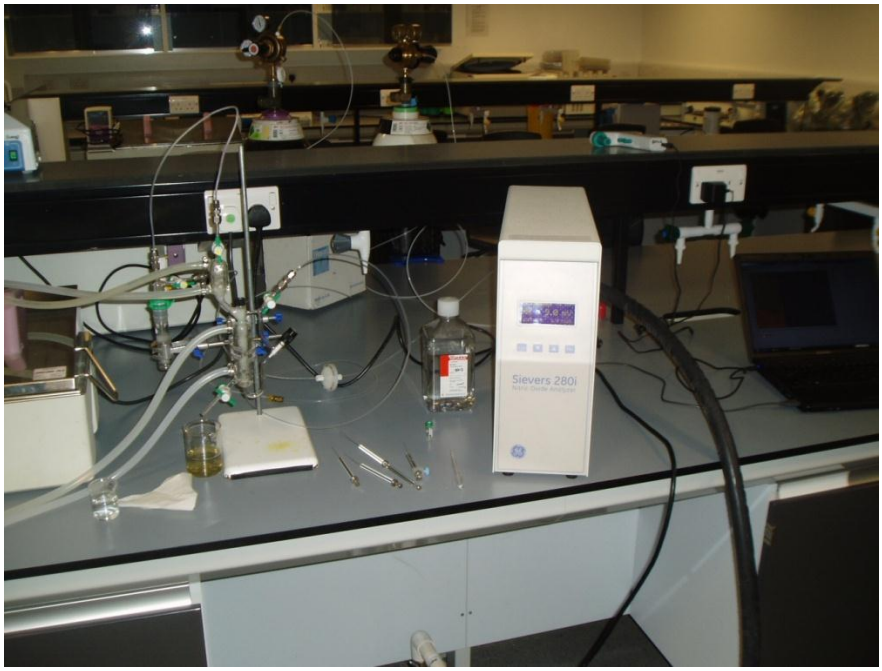


Figure 22: The Seivers 180i NOA Nitric Oxide (NO) Analyser (Analytix, Durham).

Nitric oxide can be a biomarker of cellular damage in MSCs and NO is involved in fracture healing. Corbett *et al*. (1999) hypothesised that NO isoforms, responsible for the generation of NO, are expressed during fracture healing. They reported higher levels of endothelial eNOS in cortical blood cells and osteocytes in the early stages of normal fracture repair (Corbett *et al.*, 1999; Corbett *et al.*, 1999), thus rendering the apparatus as a suitable analytical tool in this study.

2.3.5 Nitric Oxide (NO) Analytical Assay in CSE-Treated MSCs

NB: NO reacts with volatile organic compounds in the presence of sunlight to form Ozone. Ozone can cause adverse effects such as damage to lung tissue and reduction in lung function.

1. MSCs extracted from non-smokers were counted on a flow cytometer and divided into 2 numerically-equal groups (CSE treated vs. CSE untreated). The cells were seeded-out into new cultures; the inoculum being $10x10^5$ cells in each flask.

2. Cigarette smoke extract (2.5% in culture media by volume; 20 cigarettes/day) was infused into the first group; a second group contained non-infused IMDM as a control. Both groups were incubated for 7 days in 37ºC humidified 5% CO_2.

3. After incubation, cells were exposed to trypsin and centrifuged, in their respective media, in 15.0 mL centrifuge tubes. The supernatant was discarded and pellets were tapped-out, to disperse cells, which were then washed in 1.0 mL of PBS; they were again centrifuged and the resulting supernatant discarded.

4. After tapping-out the cell pellet from step 3, 100 μL of cell lysis buffer* (Bender Medsystems, Vienna) was pipetted into each of the sample tubes, which were then agitated on an orbital shaker set at 100 rpm for 60 minutes.

5. Cell suspensions were transferred to eppendorf tubes and microfuged in a benchtop microfuge set at 5000 rpm for 15 minutes at room temperature. The supernatant was discarded, leaving a gel-like liquid behind in the tube as the analyte.

6. The lysed cell analytes were then processed and analysed on the Seivers NOA 180i nitric oxide analyser.

*For 500 mL stock solution of lysis buffer; 324 mL H_2O, 10 mL 1.0 M Tris HCl, pH 8, 50 mL 10% Tx-100, 100 mL of 50% w/v in H_2O glycerol, 2.0 mL 0.5 M EDTA (pH 8.0), 13.7 mL NaCl. Store a 4ºC. Before use, add 1% 100 mM phenylmethylsulfonyl fluoride (PMSF), 100 nM vanadate, 1 mg mL^{-1} leupeptin, 0.01% 1.0 mg mL^{-1} aprotinin.

2.3.6 Cell Necrosis Assay

1. After trypsinisation, centrifugation and re-suspension, harvested cells were counted using a flow cytometer and known amounts (5×10^5) were each diluted in 900 μL of Isoton II diluent (Beckman Coulter, High Wycombe).

2. Since necrotic cells become unattached to the plastic substratum, it was important not to discard the media prior to counting. When reseeding out into the next passage, the media was changed after 24 hours to ensure viable cells had become attached to the floor of the flask.

3. 10 μL of propidium iodide (Sigma-Aldrich, Gillingham; Cat#P4864) was added to the solution and samples were immediately processed on a flow cytometer to quantify the level of necrotic cells within the cell population and were recorded as percentages. This was conducted on cells over four passages of 7days.

2.3.7 MSC Recovery Assay

1. Cells from ten non-smoking patients were cultured in IMDM and allowed to become confluent. Three experimental groups were set up as follows:

Group I (Smoking, *n*=10): CSE (2.5%; 20 cigarettes/day equiv.) was infused into the cell culture media; this was maintained through the entirety of the assay.

Group II (Recovery, *n*=10): CSE was infused for 5 days only, which was then withdrawn in subsequent cultures prior to analysis. Normal media was used for the remainder of the assay

Group III (Non-Smoking, *n*=10): Untreated MSC culture media throughout.

2. Periodic observations were made on the morphology of the cells using light microscopy; photographs were taken.

3. Cells were incubated and counted in three consecutive passages, each of which was 5d, from the baseline count. Data was normalised as percentage change from baseline count and values from each group were compared.

2.3.8 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay: In Vitro Effect of BMP-2 on Cell Viability Enhancement

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in living cells. The solubilisation solution, dimethyl sulfoxide (DMSO), is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance of this solution can be quantified by measuring at a wavelength of 570 nm by a spectrophotometer. The deeper the purple colour, the greater the cellular activity, proliferation and viability.

This assay is often used in order to analyse the viability of cells in cultured populations. It is particularly suitable where a growth factor or drug is incorporated into the culture media, necessitating relatively small quantities of the former due to costing and availability issues. Samples are analysed in 96-well uncoated plates, which are read on a spectrophotometer for absorbance determination. The MTT assay was used in order to determine the effects of bone morphogenetic protein-2 (BMP-2) on CSE-treated haematoma progenitor cells.

2.3.8.1 Cell Preparation – MTT Assay

1. Haematoma-derived progenitor cells were retrieved from the vapour phase of the N_2 dewar. Cells used were all extracted from non-smoking patients (*n*=10).

2. Cells were allowed to expand in culture until flask-confluency, which occurred in all 10 samples after 5 days of culture.

3. After the application of trypsin and re-suspending in 1.0 mL of complete media, cells were combined with an Isoton II solution containing a known quantity of Flowcount beads and counted using a flow cytometer so that suspension concentrations could be determined prior to plating-out in 96 well plates.

4. Cells were plated-out in wells at an inoculum of 3.0×10^3 per well. Three groups were created in duplicate; 24h, 48h and 72h of various amounts of BMP-2 (product no. H4791, Sigma Aldrich, Gillingham) (10 ng mL^{-1}, 100 ng mL^{-1} and 500 ng mL^{-1} per well). An additional control group that did not contain any of the BMP-2 growth factor was added to the plate.

5. All wells (including those from the control group) were subjected to CSE infusions of 2.5%, representing the circulating tobacco-derived toxins comparable to the blood of an individual smoking 20 cigarettes per day.

6. Volumes in each well were normalised to 250 μL; surrounding empty wells were filled with media only to prevent dehydration. Plates were covered and incubated at 37ºC in a humidified incubator for their respective times.

7. In order to run a separate control study, an additional plate was prepared that was composed of cells that had not been subjected to CSE. This plate had 3 groups of cells in duplicate, each containing differing amounts of BMP-2; 0, 10 and 100 ng mL^{-1}.

MTT Preparation

1. 10 mg of MTT was weighed on a balance and dissolved in 1.0 mL of dH_2O.
2. A final concentration of 1 mg mL^{-1} of MTT in IMDM was aliquoted.
3. 50 μL of the MTT solution was added to each well at the lapsed time as appropriate.
4. The reaction was allowed to continue for 4 hours in the incubator after adding the MTT compound. After 4 hours, the MTT reagent was dispensed from the plate. 100 μL of DMSO was then added to each well in order to dissolve the purple formazan product within the mitochondria of the adherent haematoma cells.
5. Plates were read at 570 nm using a microplate reader for each plate at 24h, 48h and 72h intervals once the DMSO had been added.

2.3.9 Cryopreservation of MSCs

1. After 3 passages had been subjected to sub-culture, the fracture MSCs were stored in the vapour phase of liquid nitrogen (N_2) at -160ºC for later analyses.

2. 100 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Gillingham), 900 μl of Fetal Calf Serum (FCS) and 800 μL of suspended HCs were aliquoted into a 1.8 mL labelled cryogenic storage vial (Sarstedt, Nümbrecht).

3. Vials containing the cells were placed into a small freezing vessel that contained 250 mL of isopropanol (at room temperature) and then placed in a freezer (-80°C) for 24 hours (where the temperature of isopropanol gradually reduces by 1ºC per minute), before transferring into the vapour phase of the N_2 (-160°C) reefer, for indefinite storage. Details were logged.

2.4 METHODS II: MOLECULAR ASPECTS

2.4.1 Serum Protein Determination Using SDS-PAGE

Sample Preparation:

1. The liquid component associated with the haematoma was pipetted into a 5 mL Z serum separator and clot activator tube (Vacuette, Kremsmünster) and allowed to clot for 60 minutes in a water bath at 37˚C. The haematoma samples were then centrifuged at 3500 rpm for 15 minutes at room temperature; the resulting serum was filtered using a 0.2 μm filter (Sterilin, Bargoet), pipetted into 1.0 mL aliquots and stored at -20˚C.

SDS-PAGE Assay:

2. The serum that had been extracted from the haematoma (step 1) was retrieved from the -20°C freezer and thawed in a water bath that was set at 37°C.

3. 50 μL of haematoma serum was pipetted from the sample (step 2) and dispensed into a 15 mL centrifuge tube that contained 950 μL of phosphate buffered saline (PBS). This formed a dilution of 5% serum in 95% PBS.

4. The serum/PBS solution was centrifuged at 3500 rpm for 15 minutes at 4°C to exclude any debris. Control samples, using FCS and human peripheral blood sera, were prepared in exactly the same way as the haematoma serum sample. A protein marker (SERVA, Heidelberg) was also included as a separate lysate to estimate the molecular weight of proteins in the gel.

5. 45.0 μL of each centrifuged lysate (from step5) was pipetted into an eppendorf tube that contained 45.0 μL of sample buffer (SERVA, Heidelberg) (Tris-HCl pH 6.8, 126mM, glycerol 20%, SDS 4%, bromophenol blue 0.02%). 5.0 μL of 2-mercaptoethanol (Sigma-Aldrich, Gillingham) was then added to each solution to reduce the disulphide bonds present in protein.

6. Each eppendorf tube was suspended (but not fully immersed) vertically in boiling water for 5 minutes to denature the proteins; the tubes were then placed on wet ice for 10 minutes.

7. The TV-50 electrophoresis cell (Scie-Plas, Cambridge) was fitted with a 10 cm x 10 cm precast gel-cassette (Tris-Glycine Gel 16%) (SERVA, Heidelberg). The apparatus was then filled with 290 mL of Laemmli buffer (0.25 M Tris, 1.92 M glycine and 1.0% SDS) (SERVA, Heidelberg).

8. After removal of the comb, 5.0 μL of each sample was carefully loaded into the wells of the stacking gel in a specified arrangement (see later) using a 10.0 μL pipette (Gilson, Middleton). The electrophoresis cover was placed onto the apparatus.

Electrophoresis stacking conditions:

9. The electrophoresis power pack (Consort E815, Brussels) was switched on and stacking conditions were programmed (and then ran) as follows: 10 mA, 100 V, 300W, 15 minutes.

Electrophoresis resolving conditions:

10. After the stacking cycle had finished, the electrophoresis power pack was reprogrammed (and ran) for resolving conditions as follows: 20 mA, 100 V, 300W, 120 minutes.

Gel Staining I: Coomassie Blue

11. A Coomassie blue stain was prepared by dissolving 1 *Phast R* tablet (Sigma-Aldrich, Gillingham) into 120 mL of dH_2O and 80.0 mL methanol.

12. After the gel had completely undergone electrophoresis, it was removed from the apparatus and the cassette housing was carefully removed with the supplied plastic key (SERVA, Heidelberg).

13. The gel was placed into a clean plastic container and gently washed for 1 minute in dH_2O.

14. 100 mL of Coomassie blue staining solution was poured into the container that contained the gel. It was then covered with a lid and placed on an orbital shaker, which had been set at 50 rpm for 30 minutes.

15. After 30 minutes, the Coomassie blue stain was poured away and safely discarded and the gel was washed again in two changes of dH_2O.

Gel destaining:

16. The gel was placed in 100 mL of destaining solution (60% dH_2O, 30% methanol, 10% glacial acetic acid), covered and placed on an orbital shaker set at 50 rpm for 60 minutes. This step was repeated twice.

17. The gel was then immersed in 250 mL dH_2O and left overnight to remove excess stain and was later photographed.

Gel Staining II: Silver Nitrate Staining

Polyacrylamide gels were also stained using the silver nitrate method to further improve clarity of resolution of protein bands. This method is described as follows;

1. After opening and removal from the plastic housing following electrophoresis as previously described, the gel was placed in a fixing solution of 50% dH_2O, 40% absolute ethanol and 10% glacial acetic acid for 30 minutes.

2. The gel was then immersed for 30 minutes in a sensitizing solution of 63% dH_2O, 30% ethanol, 6.8% anhydrous sodium acetate (FW. 82.03) and 0.2% sodium thiosulphate pentahydrate (FW. 248.18). 0.5 mL of 25% gluteraldehyde per 100 mL of this solution was added.

3. The gel was washed for 30 minutes in a silver solution (Sigma-Aldrich, Gillingham) containing 0.25g of silver nitrate (FW. 169.87) in 100 mL of dH_2O. 40.0 μL of 37% formaldehyde was added. After 30 minutes, the gel was washed in dH_2O for 1 minute (x2 washes).

4. A developing solution consisting of 2.5g of anhydrous sodium carbonate (FW. 105.99) dissolved in 100 mL of dH_2O was prepared with the addition of 20 μL of 37% formaldehyde. The gel was immersed in this solution for 5 minutes.

5. A stop solution was prepared using 1.5g of Na_2 EDTA (ethylenediaminetetraacetic acid) in 100 mL of dH_2O. The resolved gel was placed in the solution for 10 minutes before being washed in dH_2O (3 x 5 minute washes).

7. Preserving solution was prepared as follows; 64 mL dH_2O, 30 mL absolute ethanol and 4.0 mL glycerol; the constituents were mixed thoroughly. The gel was stored in this solution under refrigeration at 4ºC. Gels can be stored in this way for up to 2 years.

2.4.2 SDS-PAGE Gel Production:

1. On certain occasions, SDS-PAGE gels were made in the laboratory to save on cost and delivery times. The gel pouring protocol is as follows:

15% Running Gel

44.4% acrylamide/1.2% bis acrylamide	(3.38 mL)
1M Tris/HCL (pH 8.8)	(3.75 mL)
dH_2O	(2.72 mL)
10% SDS	(100 μL)
10% ammonium persulfate	(50.0 μL)
TEMED (to be added directly prior to pouring)	(5.0 μL)

Isopropanol for sealing gel

Polymerisation time = 30 minutes

5% *Stacking Gel*

44.4% acrylamide/1.2% bis acrylamide	(500 μL)
1M Tris/HCL (pH 6.8)	(625 μL)
dH_2O	(3.8 mL)
10% SDS	(50 μL)
10% ammonium persulfate	(25 μL)
TEMED (to be added directly prior to pouring)	(5.0 μL)

Polymerisation time = 30 minutes

2. The glass casting plates were filled with running gel approximately 1.0cm from the top, air bubbles were minimised, ensuring the surface was level. The top was sealed with isopropanol and left to polymerise for 30 minutes.

3. After 30 minutes, the isopropanol was discarded and the liquid stacking gel was pipetted onto the polymerised running gel before inserting comb. The gel was ready to use 30 minutes thereafter.

NB. Glass plates should be sealed with Blu-Tack® to prevent leakage.

2.4.3 Analysis of SDS-PAGE GELS via Mass Spectrometry

Unknown bands on SDS-PAGE gels can be excised from the acrylamide polymer, the gel digested to solubilise proteins, which can then be detected and given an identity, using mass spectrometry (MS). The following protocol was supplied by the Biopolymer Synthesis and Analysis Unit (BSAU) at the University of Nottingham:

1. Gel bands were manually excised and subjected to automated in-gel chemical modification of cysteine residues with dithiothreitol (dtt) and iodoacetamide followed by tryptic digestion and peptide extraction on the MassPrep robotic system (Waters, Milford).

2. The extracted peptide mixture was run on a Waters QTOF2 hybrid quadruple mass spectrometer incorporating an integrated capillary lc system. A total of 5μL of the digest was introduced via an autosampler.

3. This was initially loaded onto a small C18 packed pre-column for desalting and then products of the tryptic digest were eluted onto an analytical capillary C18 column (100mm X 0.75mm id). The lc system incorporated a flow splitting device to give a final flow through the column of 200 nL per minute.

4. A solvent gradient was run over a total of 1 hour to elute peptides from the column and re-equilibrate prior to loading the next sample. Where samples were observed to contain high levels of peptides a blank run was usually incorporated between sample runs to establish that there was no significant carry-over of one sample to the next.

5. Eluted peptides from the analytical column were directly submitted into the mass spectrometer via a nanosprayer device attached to the outflow from the lc system and operating at 3kV. In addition, a reference solution containing a peptide of known mass was sprayed into the mass spectrometer from a separate sprayer. This ion source was sampled at regular intervals throughout the run to assist in maintaining accurate mass measurements of the ionised peptides from the analyte spray.

6. Data-dependent switching was incorporated so that whenever a peptide with an associated charge of 2+ or 3+ was detected above a pre-set threshold signal, the mass spectrometer would automatically switch to ms-ms mode to generate fragmentation data from the detected peptide. The software was set up to scan over multiple channels in order to simultaneously fragment up to 3 co-eluting peptides and collect the fragmentation data from each one individually. A preset range of collision voltages was set up in the method in order to fragment each peptide as efficiently as possible.

7. Raw data files were analysed using MassLynx 4.0 (incorporating BioLynx) and ProteinLynx Globalserver 2 (Waters, Milford) in order to assess the identities of proteins present in the digest. The peak list files generated from PLGS2 analysis were also used in an alternative search program accepting this format of data file (Matrix Science, Boston). In addition some fragmentation data was analysed manually and de novo sequencing carried out on selected peptides.

2.4.4 TGF-β and IL-6; Intracellular Markers in MSCs

Immunocytochemical analysis of cytoplasmic acute-phase proteins, using flow cytometry, qualitatively detects the presence of these within the haematoma cells. Probing permeated cells with markers specific for TGF-β and IL-6 was undertaken. If cultured haematoma cells are expressing these cytokines, which are involved in fracture healing and are known to be secreted by MSCs, then further analyses could be conducted to measure them *quantitatively* in order to establish differences between smokers' and non-smokers' cells.

Cell Preparation

1. Confluent MSC cultures from non-smoking patients were trypsinised, centrifuged and washed and re-suspended in 1.0 mL of PBS. Cell cultures were each divided into two groups; cigarette smoke extract (CSE) was infused into one group and the other left untreated. MSC concentrations were determined on a flow cytometer.

2. Equal concentrations of 10×10^5 MSCs were calculated in each sample and pipetted into 15.0 mL centrifuge tubes.

3. Suspensions were centrifuged at 1000 rpm for 10 minutes at room temperature; the supernatant was discarded before adding 100 μL of ice-cold methanol (-20ºC). Samples were incubated at -20ºC for 10 minutes.

4. To permeablise cells, 100 μL of PBS with 0.1% triton X-100 was added to each sample, which were then incubated at room temperature for 15 minutes.

5. A further 1.0 mL of PBS containing 0.1% triton X-100 was added to each sample, which were then centrifuged at 2000 rpm for 5 minutes; 4ºC.

6. The supernatant was discarded and the cell pellet tapped-out in order to disperse the permeablised cells.

Intracellular Staining (FITC)

7. 5.0 μL of primary monoclonal antibody conjugated to FITC (Sigma-Aldrich, Gillingham), mouse, anti-human, specific to TGF-β or IL-6 (as appropriate) was added, separately, to each sample and incubated for 45 minutes at 4ºC in a refrigerator.

8. After the incubation period with the primary antibody specific to either TGF-β or IL-6, cells were washed by centrifuging at 1200 rpm for 10 minutes in 1.0 mL of PBS and then re-suspended in 1 mL of PBS

9. 50 μL of secondary polyclonal antibody (diluted 1:30 with 3% bovine serum albumin (BSA) and 97% PBS) was added to each sample, which were then incubated for 30 minutes at room temperature in a dark place.

10. Cells were washed as step 8 in 1.0 mL of PBS before re-suspending in ice-cold (~4ºC) PBS containing 3% BSA and 1% acetone.

11. The protein-labelled cells were analysed using a flow cytometer; data was filed and stored.

2.4.5 VEGF and IL-6 Quantitation via ELISA in Haematoma Serum and Lysed Fracture MSCs: A Preliminary Study

ELISA kits used for the following assays were the VEGF-A (EK3099-A) (Arcus Biologicals, Modena) and IL-6 ELISA (BMS213INSTCE) (Bender Medsystems, Vienna).

1. Cells from smokers (*n*=4) and non-smokers (*n*=5) were trypsinised and suspended in 1.0 mL of complete media and were diluted 1:10 with the supplied prepared assay buffer, which is composed of animal serum with buffer and preservative (20μL sample:180 μL buffer:). Haematoma serum was diluted 50:50 with the prepared assay buffer. This was the recommended dilution in the manufacturer's datasheet.

2. 20 μL 1N HCL was added to 200 μL of pre-diluted sample, which was mixed and incubated for 1 hour at room temperature. This reaction was then neutralised by the addition of 20 μL of 1 M Na OH.

3. All reagents were mixed thoroughly without foaming before use.

4. The number of microwell strips that were required to test the desired number of samples was determined, plus the appropriate number of wells required for running blanks and standards. Each sample, standard, blank and optional control sample were all assayed in triplicate.

5. The required amount of microwell strips were washed 3 times with 300 mL of wash buffer per well; all microwell contents were aspirated between washes, taking care not to scratch the base of the coated microwells.

6. After the final wash, the microwell strips were tapped on an absorbent disposable pad or paper towel to remove excess wash buffer. The microwell strips were used immediately after washing, taking care not to allow the wells to dry.

ELISA standard preparation (VEGF and IL-6)

1. 100μL of assay buffer, in duplicate, was added to all standard wells. Standard solutions were prepared by pipetting 100μL of the standard, in duplicate, into wells A1 and A2; the contents mixed by repeated aspiration and ejection. 100μL was transferred from well A1 to well B1 and from A2 to B2, respectively. This process was repeated four more times, until wells G1 and G2 were filled; 100 μL was discarded from each of these wells. The two rows of standard dilutions ranged from 30.0 to 0.5 ng mL^{-1}.

2. 100μL of assay buffer, in duplicate, was added to the blank wells.

3. 100 μL of each pre-treated sample, in duplicate, was added to each of the designated wells.

4. Horse radish peroxidase (HRP) conjugate was prepared as per the datasheet and 50μL of diluted HRP conjugate was added to all wells.

5. The plate was covered with a cover (supplied) and incubated at room temperature for 4 hours on an orbital shaker set at 50 rpm.

6. The plate cover was removed and the wells were emptied. The microwell strips were washed 3 times.

7. 100μL of tetramethylbenzidine (TMB) substrate solution was pipetted into all wells, including the blank wells.

8. The microwell strips were incubated at room temperature for 10 minutes, avoiding exposure to light. The colour development was monitored; 100μL of the stop solution was added when the highest standard developed a dark blue colour. The stop solution causes a change in colour from blue to yellow.

9. The results were read immediately on a plate reader set at 420 nm.

2.4.6 Bone Alkaline Phosphatase (b-ALP) Quantitation in Fracture Serum

Skeletal, or bone-specific alkaline phosphatase (b-ALP) is a tetrameric glycoprotein found on the surface of osteoblastic cells and is therefore involved in the laying-down of the bone matrix as seen in fracture repair. Although the precise function of b-ALP is not fully understood, it is accepted that it is a useful biochemical marker of bone turnover (Frost-Pineda *et al.*, 2010). An assay was performed to measure the amounts of b-ALP present in the fracture haematoma serum collected from patients involved in this study and data was compared between smokers and non-smokers in respect of this biomarker. The details of the assay (Horiba datasheet) are summarised as follows:

$$\text{p-Nitrophenylphosphate} + H_2O \xrightarrow{[alp]} \text{Phosphate} + \text{p-Nitrophenol}$$

1. Processed fracture haematoma serum samples were retrieved from the -80ºC freezer and divided into non-smoking (*n*=10) and smoking (*n*=10) patient groups.

2. The samples were numbered accordingly (1-10 (smokers), 11-20 (non-smokers) and allowed to thaw at room temperature prior to placing on wet ice ready for analysis.

3. The biochemical analyser (Horiba ABX Pentra DF-DX 120, Tokyo), was prepared as per the manufacturer's specifications.

4. *ALP Reagent 1* was prepared as follows: 2-amino-2-methyl-1-propanol (pH 10.4) - 440 mMol L^{-1}, magnesium sulphate - 2.0 mMol L^{-1}, zinc sulphate - 1.25

mmol L^{-1}, HEDTA (hydroxyethylethylenediaminetriacetic acid) - 2.5 mMol L^{-1}, sodium azide - < 1g L^{-1}.

5. *ALP Reagent 2* was prepared as follows: p-nitrophenylphosphate - 80 mMol L^{-1}, sodium azide - < 1 g L^{-1}.

6. The required volume of *ALP Reagent 1* was transferred into a 15 mL test tube.

7. The required volume of *ALP Reagent 2* was transferred in a 10 mL test tube.

8. The reagents were placed in the rack in the refrigerated ABX Pentra 400 reagent compartment ready for use.

9. For calibration, ABX Pentra MultiCal, Ref. A11A01652 was used.

10. The ABX Pentra Ref. A11A01653 was used as a control.

11. Reagent 1 was placed in position 1 of one available sector of the analyser.

12. Reagent 2 was placed in position 2 of same selected sector of the analyser.

13. 100 μL of each sample, combined with 400 μL dH_2O (1:4) was loaded into Eppendorf tubes which were then placed into the test wells ready for analysis.

14. The machine generated the results and this data was recorded and analysed statistically using a Microsoft Excel spread sheet. Datasheets available overleaf.

ABX Pentra

Form-0846 Rev. 2
Use in reagent rack
2007/06/08
A93A00012N EN

A11A01626

26 ml
6.5 ml
HORIBA ABX
BP 7290
34184 Montpellier- cedex 4 - France
S.A.S. au capital de 41.700.000 € - RCS Montpellier 328 031 042 - SIRET 328 031 042 000 42 - APE 332 B Latest version documents on www.horiba-abx.com

Diagnostic reagent for quantitative *in-vitro* determination of Alkaline Phosphatase (ALP) in serum or plasma.

Clinical Interest (1,2)

Alkaline phosphatase (ALP), an hydrolytic enzyme acting optimally at alkaline pH, exists in blood in numerous distinct forms which originate mainly from bone and liver, but also from other tissues as kidney, placenta, intestine, testes, thymus, lung and tumors. Physiological increases are found during bone growth in childhood and in pregnancy, while pathological increases are largely associated with hepatobiliary and bone diseases. In hepatobiliary disease they indicate obstruction of the bile ducts as in cholestasis caused by gall stones, tumors or inflammation. Elevated activities are also observed in infectious hepatitis. In bone diseases elevated ALP activities originate from increased osteoblastic activity as in Paget's disease, osteomalacia (rickets), bone metastases and hyperparathyroidism.

Method

Kinetic photometric test, according to the International Federation of Clinical Chemistry (IFCC).
(ALP = Alkaline Phosphatase)

Reagents

ABX Pentra ALP CP is ready-to-use.
ABX Pentra ALP CP should be used according to this reagent notice. HORIBA ABX cannot guarantee its performance if used otherwise.

Handling

Transfer the required volume of Reagent 1 in a container 15, 10 or 4 ml.
Transfer the required volume of Reagent 2 in a container 10 or 4 ml.
Reagent 1 and Reagent 2 should be placed on the same reagent rack sector A, B or C (see diagram below, sector A is taken as an example).
Reagent 1: 2-Amino-2-methyl-1-propanol pH 10.4 440 mmol/l
Magnesium sulphate 2.0 mmol/l
Zinc sulphate 1.25 mmol/l
HEDTA 2.5 mmol/l
Sodium azide < 1 g/l
Reagent 2: p-Nitrophenylphosphate 80 mmol/l
Sodium azide < 1 g/l
p-Nitrophenylphosphate + H_2O Phosphate + p-Nitrophenol
ALP
Place Reagent 1 in position 1 of one available sector using either:
• Reagent container 15 ml
• Reagent container 10 ml + its specific adaptor
• Reagent container 4 ml + its specific adaptor
Place Reagent 2 in position 2 of same selected sector using either:

• Reagent container 10 ml
• Reagent container 4 ml + its specific adaptor
Place the reagent rack in the refrigerated ABX Pentra 400 reagent compartment.

Calibrator

For calibration, use:
ABX Pentra MultiCal, Ref. A11A01652 (not included)
10 x 3 ml (lyophilisate)

Control

For internal quality control, use:
ABX Pentra N Control, Ref. A11A01653 (not included)
10 x 5 ml (lyophilisate)
ABX Pentra P Control, Ref. A11A01654 (not included)
10 x 5 ml (lyophilisate)
Each control should be assayed daily and/or after each calibration.
ABX Pentra ALP CP
Reagent 2
ABX Pentra ALP CP
Reagent 1

ABX Pentra

The frequency of controls and the confidence intervals should correspond to laboratory guidelines and country-specific directives.
The results must be within the range of the defined confidence limits.
Each laboratory should establish a procedure to follow if the results exceed these confidence limits.

Materials required but not provided

• Automated clinical chemistry analyser
• Standard laboratory equipment.

Specimen

• Serum.
• heparin plasma.

Loss of activity within 2 - 3 days at 15 - 25 °C < 10 %.

Reference range (4,5)

Adults:
Children:

Storage and Stability

Reagents, in unopened cassettes, are stable up to the expiry date on the label if stored at 2-8 °C protected from light and contamination is avoided.
Stability after opening : refer to the paragraph ''Performance on ABX Pentra 400''.
Do not freeze the reagents.

Assay Procedure

Test instructions for other automated systems than ABX Pentra 400 are available on request.

Waste Management

1. Please refer to local legal requirements.
2. This reagent contains sodium azide (0.95 g/l) as a preservative. As sodium azide may react with lead or copper to form explosive metal azides, this reagent should be disposed of by flushing with copious amounts of water.

General Precautions

1. This reagent is for professional *in-vitro* diagnostic use only.
2. Do not swallow! Avoid contact with skin and mucous membranes.
3. During reaction p-nitrophenol is produced which is poisonous when inhaled, swallowed or absorbed through skin. If the reaction mixture comes in contact with skin or mucous membranes wash copiously with water.
4. Take the necessary precautions for the use of laboratory reagents.
5. The reagent cassettes are disposable and should be disposed of in accordance with the local legal requirements.

6. Please refer to the MSDS associated with the reagent.

Performance on ABX Pentra 400

The performance data listed below have been obtained on the ABX Pentra 400 analyser.
Number of tests: 125 tests
Reagent Stability:
Use fresh reagent each day. Discard the remaining reagent in container after use. Once open, the cassette is stable for 29 days.
Sample volume: 4 ⌈l/test
Detection limit:
The detection limit is determined according to the Valtec protocol (6) and equals 6 U/l.
Accuracy and Precision:
• Repeatability (within-run precision)
3 specimens of low, medium and high concentration and 2 controls are tested 20 times according to the recommendations found in the Valtec protocol (6).
Stability : 7 days at 4 - 8 °C
2 months at - 20 °C
37 °C
Women [U/l] 35 - 104
Men [U/l] 40 - 129
37 °C
1 day [U/l] < 250
2 to 5 days [U/l] < 231
6 days to 6 months [U/l] < 449
7 months to 1 year [U/l] < 462
1 to 3 years [U/l] < 281
4 to 6 years [U/l] < 269
7 to 12 years [U/l] < 300
13 to 17 years (female) [U/l] < 187
13 to 17 years (male) [U/l] < 390
Mean value U/l CV %
Normal control 90.79 1.27
Pathological control 252.68 0.62
Specimen 1 28.05 3.98
Specimen 2 54.88 2.42
Specimen 3 430.87 0.84

ABX Pentra

• Reproducibility (run-to-run precision)
2 specimens of medium and high levels and 2 controls are tested in duplicate for 20 days (2 series per day) according to the recommendations found in the NCCLS, EP5-A protocol (7).
Linearity and Measuring Range:
The reagent linearity is determined according to the recommendations found in the NCCLS, EP6-P protocol (8).
Low linearity: 6 U/l
High linearity: 1500 U/l, with automatic post-dilution: 6000 U/l.
Correlation:
100 patient samples are correlated with a commercial reagent taken as reference according to the recommendations found in the NCCLS, EP9-A2 protocol (9).
The equation for the allometric line obtained is:
Y = 0.99 x - 1.22 with a correlation coefficient r^2 = 0.99.
Interferences:
Calibration stability:
The calibration stability is 7 hours.
Note: A recalibration is recommended when reagent lots change, and when quality control results fall outside the range established.
Application release: 7.xx

2.4.7 Albumin Quantitation in Fracture Serum

Albumin is a main constituent of plasma protein and has a vital role in the maintenance of osmotic pressure. It is a key mediator for the fixation and transport of a plethora of circulatory products. It is known that albumin serum constitutes a predictive factor in the altered levels of bilirubin, calcium and hormones due to an impaired hepatic function and inflammatory processes. An increase in albumin is indicative of dehydration, whereas decreases may represent malnutrition, synthesis alteration, liver disease and the acute-phase response (Doumas and Peters, 1997; Nolen *et al.*, 2013).

From Horiba datasheet: Colorimetric determination is via bromoscol green (BCG). At pH 4.2 the BCG selectively fixes itself to albumin, colouring it blue. The biochemical analyser (Horiba, Montpellier) can quantitatively determine in serum via colorimetry, as per the following methodology;

1. Steps 1-3 were repeated as for the b-ALP protocol, as previous.

2. The ABX Pentra Albumin ready-to-use reagent was employed. The solution is comprised of the following constituents:

- Succinate acid: 58 mMol L^{-1}
- Bromocresol green: 0.14 g L^{-1}
- Brij 35: 7 mL L^{-1}

3. The reagent was placed in the relevant compartment in the machine. Commercially available calibrator (ABX Pentra Multical) and control (ABX Pentra N and P control) were also used alongside the albumin-binding reagent.

4. Samples were processed on the analyser as previous protocol. Datasheets available overleaf.

ABX Pentra

Form-0846 Rev. 2
Serum/Plasma
2007/08/27
A93A01042I EN

A11A01623

19 ml
4.5 ml
HORIBA ABX
BP 7290
34184 Montpellier- cedex 4 - France
S.A.S. au capital de 41.700.000 € - RCS Montpellier 328 031 042 - SIRET 328 031 042 000 42 - APE 332 B **Latest version documents on www.horiba-abx.com**

Diagnostic reagent for quantitative *in-vitro* determination of Albumin (ALB) in serum and plasma by immunoturbidimetric assay.

Clinical Interest

Albumin constitutes a prediction factor in the transport alteration of bilirubin, calcium and hormones related to hepatic dysfunction and/or inflammation.
ABX Pentra Micro-albumin CP is a immunoturbidimetric assay developed to accurately measure ALB concentration in serum and plasma samples.

Method

When an antigen-antibody reaction occurs between ALB in a sample and anti-ALB antibody agglutination results. This agglutination is detected as an absorbance change, with the magnitude of the change being proportional to the quantity of ALB in the sample. The actual concentration is then determined by interpolation from a calibration curve prepared from calibrators of known concentration.

Reagents

ABX Pentra Micro-albumin CP is ready-to-use.
1. After measurements are taken, reagent cassettes should remain in ABX Pentra 400 refrigerated tray.
2. Care should be taken not to interchange the caps of the reagent cassettes.
3. Reagents with different lot numbers should not be interchanged or mixed.
4. **ABX Pentra Micro-albumin CP** should be used according to this reagent notice. HORIBA ABX cannot guarantee its performance if used otherwise.

Handling[a]

Remove both caps of the cassette. If present, remove foam by using a plastic pipette.
Position the respective protective cap, ref. GBM0969 on R1 and Ref. GBM0970 on R2 and place in the refrigerated ABX Pentra 400 reagent compartment.

Calibrator

For calibration, use:
ABX Pentra Protein Cal, Ref. A11A01698 (not included)
4 x 1 ml
Reagent 1: Buffer solution
Glycine buffer solution
Reagent 2: Antiserum
Anti-human ALB antibody (goat)
containing 1.2-2.4 units per 1ml
a. Modification from index H to I: new handling.

Control

For internal quality control, use:
ABX Pentra Protein Control L/H, Ref. A11A01700 (not included)
2 x 1 ml + 2 x 1 ml (Only the low control is titrated)
Each control should be assayed daily and/or after a calibration.
The frequency of controls and the confidence intervals should correspond to laboratory guidelines and country-specific directives.
The results must be within the range of the defined confidence limits.
Each laboratory should establish a procedure to follow if the results exceed these confidence limits.

Materials required but not provided

• Automated clinical chemistry analyser
• **ABX Pentra Sample diluent CP**, Ref. A11A01662, 99 ml
• Standard laboratory equipment.

Specimen

• Serum.
• Plasma.
After sampling, the test should be performed without delay. If the test can not be done immediately, the sample should be placed in a tightly sealable container and stored at -20 °C. Repeated freezing and thawing should be avoided.

Reference range

35 - 52 g/l.
Because values could vary according to the age, the diet, the gender and the geographic repartition, we recommend that each laboratory establishes its own reference range.

ABX Pentra

Storage and Stability

Reagents, in unopened cassettes, are stable up to the expiry date on the label if stored at 2-10 °C and protected from light.
Stability after opening : refer to the paragraph ''Performance on ABX Pentra 400''.

Assay Procedure

Test instructions for other automated systems than ABX Pentra 400 are available on request.

Waste Management

1. Please refer to local legal requirements.
2. This reagent contains less than 0.1 % of sodium azide as a preservative. As sodium azide may react with lead and copper to form explosive metal azides, this reagent should be disposed of by flushing with copious amounts of water.

General Precautions

1. This reagent is for professional *in-vitro* diagnostic use only.
2. Diagnosis should only be made after taking clinical symptoms and the results of other tests into consideration.
3. Specimens should be treated as potentially infectious (HIV, Hepatitis B virus, Hepatitis C virus, etc.) and handled with appropriate caution.
4. The reagent cassettes are disposable and should be disposed of in accordance with the local legal requirements.
5. Please refer to the MSDS associated with the reagent.

Performance on ABX Pentra 400

The performance data listed below have been obtained on the ABX Pentra 400 analyser.
Number of tests: 150 tests
On board Reagent Stability[a]:
Once opened, the reagent cassette placed in the refrigerated ABX Pentra 400 reagent compartment is stable for 35 days.
Sample volume: 12 ⌈l/test
Detection limit:
The detection limit is determined according to the Valtec protocol (2)

and equals 1 g/l.

Accuracy and Precision:

• Repeatability (within-run precision)

3 specimens of low, medium and high concentration and 1 control are tested 20 times according to the recommendations found in the Valtec protocol (2).

• Reproducibility (run-to-run precision)

2 specimens of low and high levels and 1 control are tested in duplicate for 20 days (2 series per day) according to the recommendations found in the NCCLS, EP5-A protocol (3).

Linearity and Measuring Range:

The reagent linearity is determined according to the recommendations found in the NCCLS, EP6-P protocol (4).

Low linearity: 1 g/l

High linearity: 67.7 g/l, with automatic post-dilution: 677 g/l.

Correlation:

100 patient samples are correlated with a commercial reagent taken as reference according to the recommendations found in the NCCLS, EP9-A2 protocol (5).

The equation for the allometric line obtained is:

$Y = 1.17 x - 3.07$ with a correlation coefficient $r^2 = 0.96$.

Interferences:

Prozone effect:

No excess of antigen has been detected up to a critical concentration of 450 g/l.

Calibration stability:

The reagent is calibrated on Day 0. The calibration stability is checked by testing 2 control specimens.

The calibration stability is 23 days.

Note: A recalibration is recommended when reagent lots change, and when quality control results fall outside the range established.

Application release[b]: 4.xx

a. Modification from index H to I: new on board reagent stability.

Mean value g/l CV %

Normal control 40.32 0.90

Specimen 1 15.50 1.65

Specimen 2 37.52 1.07

Specimen 3 50.18 1.19

Mean value g/l CV %

Normal control 42.54 3.36

Specimen 1 19.83 5.84

Specimen 2 49.20 3.18

Haemoglobin: No significant influence is observed up to 260 ⌈mol/l

Triglycerides: No significant influence is observed up to 7 mmol/l

Total Bilirubin: No significant influence is observed up to 530 ⌈mol/l

Direct Bilirubin: No significant influence is observed up to 720 ⌈mol/l

b. Modification from index H to I: suppression of minor index.

2.4.8 Total Protein Quantitation in Fracture Serum

The clinical significance of gross total protein changes suggests a presence of disease states. The test is normally performed in conjunction with additional biochemical tests such as serum albumin, liver function and protein electrophoresis. The albumin/globulin ratio can be calculated to procure more rigorous details regarding a patient's condition. Increased levels of total protein are associated with dehydration, lymphoma and chronic liver disease, whilst terminal liver disease and renal failure are synonymous of decreased amounts (Passing and Bablok, 1983; Nolen *et al.*, 2013).

From Horiba datasheet: The principles of the total protein method are concerned with the reaction of peptide bonds of protein with copper II ions in alkaline solution to form a blue-violet complex; known as *the biuret reaction*. The colour formed is proportional to the protein concentration.

1. The samples were prepared as the two previous biochemical assays.
2. The ABX Pentra total protein 100 CP ready-to-use reagent was obtained, which consisted of the following constituents:

- Copper II sulphate 12 mMol L^{-1}
- Potassium sodium tartrate 32 mMol L^{-1}
- Potassium iodide 30 mMol L^{-1}
- Sodium hydroxide 600 mMol L^{-1}

3. The reagent was placed in the relevant compartment in the machine. Commercially available calibrator (ABX Pentra Multical) and control (ABX Pentra N and P control) were also used alongside the albumin-binding reagent. Samples were processed on the analyser as previous protocol. Datasheet available overleaf.

ABX Pentra

Form-0846 Rev. 2
2007/09/06
A93A00252L EN
S.A.S. au capital de 41.700.000 € - RCS Montpellier 328 031 042 - SIRET 328 031 042 000 42 - APE 332 B Latest version documents on www.horiba-abx.com

A11A01669

61 ml

HORIBA ABX
BP 7290
34184 Montpellier - cedex 4 - France

Diagnostic Reagent for quantitative *in-vitro* determination of Total Proteins in serum by colorimetry.

Clinical Interest

Blood plasma is a concentrated solution of proteins, 60% of which is albumin. Considered in their entirety, plasma proteins perform very different tasks ranging from the maintenance of oncotic pressure to the transport of various molecules. They are involved in the complex mechanisms of blood coagulation and immunological reactions against antibodies. Enzymes, contained at low levels, constitute one group of the various proteins. An increase in their activity is a reliable indicator for cell injuries.
The variations of the global level of proteins thus present a value of diagnostic orientation, which however should be completed with a more specific balance.
Hypoproteinaemias reflect low levels of albumin linked to an abnormal renal protein escape, a protein synthesis defect (hepatic insufficiency) or a deficiency disease.
Hyperproteinaemias are notably observed in connection with dehydration symptoms, but they can also result from dysglobulinaemia or a myeloma.

Method

Biuret reaction
End-point method
In the presence of copper salts, serum proteins form a coloured complex in an alkaline environment.

Reagents

ABX Pentra Total Protein CP is ready-to-use.
ABX Pentra Total Protein CP should be used according to this reagent notice. HORIBA ABX cannot guarantee its performance if used otherwise.

Handling

Remove cap of the cassette. If present, remove foam by using a plastic pipette. Position the protective cap, ref. GBM0969 and place in the refrigerated ABX Pentra 400 reagent compartment.

Calibrator

For calibration, use:
ABX Pentra MultiCal, Ref. A11A01652 (not included)
10 x 3 ml (lyophilisate)
Reagent: Potassium iodide 6 mmol/l
Potassium sodium tartrate 21 mmol/l
Copper sulphate 6 mmol/l
Sodium hydroxide 58 mmol/l

Control

For internal quality control, use:
ABX Pentra N Control, Ref. A11A01653 (not included)
10 x 5 ml (lyophilisate)
ABX Pentra P Control, Ref. A11A01654 (not included)

10 x 5 ml (lyophilisate)
Each control should be assayed daily and/or after each calibration.
The frequency of controls and the confidence intervals should correspond to laboratory guidelines and country-specific directives.
The results must be within the range of the defined confidence limits.
Each laboratory should establish a procedure to follow if the results exceed these confidence limits.

Materials required but not provided

• Automated clinical chemistry analyser
• Standard laboratory equipment

Specimen

• Serum

Reference range(8)

We recommended that each laboratory establishes its own reference range.
Ambulatory patients: 64 - 83 g/l
6.4 - 8.3 g/dl
Stationary patients: 60 - 78 g/l
6.0 - 7.8 g/dl

ABX Pentra

S.A.S. au capital de 41.700.000 € - RCS Montpellier 328 031 042 - SIRET 328 031 042 000 42 - APE 332 B Latest version documents on www.horiba-abx.com

Storage and Stability

Reagents, in unopened cassettes, are stable up to the expiry date on the label if stored at 2-8° C and protected from light.
Stability after opening: refer to the paragraph "Performance on ABX Pentra 400".

Assay Procedure

Test instructions for other automated systems than ABX Pentra 400 are available on request.

Waste Management

Please refer to local legal requirements.

General Precautions

1. Reagent, for professional *in-vitro* diagnostic use only.
2. The reagent cassettes are disposable and should be disposed of in accordance with the local legal requirements.
3. Please refer to the MSDS associated with the reagent.

Performance on ABX Pentra 400

The performance data listed below have been obtained on the ABX Pentra 400 analyser.
Number of tests: 300 tests.
On board Reagent Stability:
If the ABX Pentra Total Protein CP cassette is left on board the instrument at all times, the cassette is stable for 17 days.
Sample volume: 2 ⌈l/test
Detection limit:
The detection limit is determined according to the Valtec protocol (4) and equals 1.56 g/l.
Accuracy and Precision:
• Repeatability (within-run precision)
3 specimens of low, medium and high concentration and 2 controls are tested 20 times according to the recommendations found in the Valtec protocol (4).
• Reproducibility (run-to-run precision)
2 specimens of low and high levels and 2 controls are tested in duplicate for 20 days (2 series per day) according to the recommendations found in the NCCLS, EP5-A protocol (5).
Linearity and Measuring Range:
The reagent linearity is determined according to the recommendations found in the NCCLS, EP6-P protocol (6).
Low linearity: 1.56 g/l
High linearity: 100 g/l, with automatic post-dilution: 300 g/l.

Correlation:
100 patient samples are correlated with a commercial reagent taken as reference according to the recommendations found in the NCCLS, EP9-A2 protocol (7).
The equation for the allometric line obtained is:
Y = 1.05 x - 2.6 with a correlation coefficient r = 0.9919.
Interferences:
Calibration stability[a]:
The calibration stability is 1 day.
Note: A recalibration is recommended when reagent lots change, and when quality control results fall outside the range established.
Application release: 4.xx

Warning

It is the user's responsibility to verify that this document is applicable to the reagent used.

Reference

1. Bernard S. Biochimie Clinique. Edition Maloine, Paris (1982), 5, 135.
2. Gornall A. et al., Determination of serum proteins by means of the biuret reaction, J. Biol. Chem., 177, (1949), 751.
3. Weichselbaum P.E., An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma, Am. J. Path, 16, (1946), 40.
4. Vassault A., Grafmeyer D. Naudin C. et al., Protocole de validation de techniques (document B), Ann. Biol. Clin., 1986, 44, 686-745.
5. Evaluation of Precision Performance of Clinical Chemistry Devices, Approved Guideline, NCCLS document EP5-A, Vol. 19, No. 2, february 1999.

Mean value g/l CV %
Normal control 51.6 1
Pathological control 51.7 1.2
Specimen 1 42.3 1.1
Specimen 2 60.6 0.9
Specimen 3 79.3 0.7
Mean value g/l CV %
Normal control 52.5 2.48
Pathological control 51.8 2.35
Specimen 1 44.9 2.79
Specimen 2 66.69 2.17

Haemoglobin: No significant influence is observed up to 72.5 ⌠mol/l
Triglycerides: No significant influence is observed up to 4.79 mmol/l
Total Bilirubin: No significant influence is observed up to 616 ⌠mol/l
Direct Bilirubin: No significant influence is observed up to 616 ⌠mol/l

a. Modification from index K to L: Modification of calibration stability.

6. Evaluation of the Linearity of Quantitative Analytical Methods, Proposed Guideline, NCCLS document EP6-P, Vol. 6, No. 18, september 1986.
7. Method Comparison and Bias Estimation Using Patient Samples, Approved Guideline, 2nd ed., NCCLS document EP9-A2, Vol. 22, No. 19, 2002.
8. Tietz, N.W., Clinical guide to laboratory tests, 3rd Ed., (W.B. Saunders eds. Philadelphia USA), (1995), 518.

ABX Pentra

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2.4.9 Statistical Data Analysis

The datasets generated by the experimental procedures were subjected to statistical analysis to gauge the level of significance. The statistical methodologies employed in this study were the Mann-Whitney U-test for non-parametric data and the Student's *t*-test and the one-way analysis of variance test (ANOVA) for the parametric data.

2.4.9.1 The Mann-Whitney U-test

This is a non-parametric test to a hypothesis about the difference in the medians of two populations using unpaired, independent data samples. The U value is calculated and then compared to the tabulated U value obtained from a published table. The null hypothesis (H_0) is tested directly by assuming it is true and a conclusion is reached to either reject H_0 or not to reject H_0. The alternative hypothesis is two-sided. If the calculated U value is less or equal to (≤) the tabulated U value, then H_0 is rejected. If the calculated U value is more than (>) the tabulated U value, then H_0 is not rejected. The calculated U value in the Mann-Whitney U-test is calculated using the following formula:

$$\mathbf{U_1 = n_1 n_2 + 0.5\, n_1\, (n_1 + 1) - R_1}$$

$$\mathbf{U_2 = n_1 n_2 + 0.5\, n_2\, (n_2 + 1) - R_1}$$

Whereby *n*1 and *n*2 are the sizes of the two samples; R1 and R2 are the sums of the ranks of observations in the first and second sample respectively. The Mann-

Whitney U test was used in the current study to calculate the statistical significance of data where differences were tested between two conditions (i.e., non-smokers and smokers) and using individual participants in each group. The test can also be applied when *n* is unequal between groups and if the data is not normally distributed (Field, 2009).

2.4.9.2 Student's *t*-Test

In the parametric experiments in the study where only one comparison was made (e.g., comparing treated cells with non-treated cells), the Student's *t*-Test was applied. The Student's *t*-Test was introduced in 1908 by William Sealy Gosset; 'Student' being a pen name. The *t*-Test interrogates the statistical hypothesis which follows a Student's *t* distribution if the null hypothesis is accepted. It can be used to determine if two sets of data are significantly different from each other, and is applied when the test statistic would follow a normal distribution. Paired samples *t*-Tests typically consist of a group of matched pairs of units that have been tested twice, which are termed 'repeated measures'. The *t*-Test is employed where subjects are tested prior to a treatment, as in the CSE studies, when cells from the same patient are tested once more after the infusions of the extract. By comparing the same patient's results before and after treating, each individual is effectively providing their own control. In this way, the correct rejection of the null hypothesis can become much more likely, with statistical power increasing due to the elimination of the random inter-variation amongst different patients (Field, 2009).

2.4.9.3 Analysis of Variance (ANOVA)

The analysis of variance method was applied to data which was normally distributed and was parametric; i.e., whereby an individual sample generated multiple datasets. Where there was more than one comparison, as in the experiments involving differing BMP-2 concentrations, a one-way ANOVA was employed. The ANOVA test is a collection of statistical models that are applied to configure differences between group means and their associated intervention(s), including variation within and between groups. With the ANOVA method, the observed variance in a specific variable is divided into components attributable to diverse sources of variation. In its simplest form, ANOVA affords a statistical test of whether or not the means of several groups are equal and applies a *t*-test to more than two groups. Undertaking multiple dual-sample *t*-Tests in one assay would result in an increased chance of committing a type I error. Type I errors occur when H_0 is true, but is rejected, declaring something that is absent, or claiming a false result. A type I error may be compared with a *false positive* in tests where a single condition is analysed. The ANOVA test is therefore justified in comparing three or more means (groups or variables) for showing statistical significance (Field, 2009).

3 CHAPTER 3

RESULTS

3.1 CLINICAL OBSERVATIONS

Patient notes were obtained in order to ascertain any clinical issues that arose during the fracture healing process. The following micrographs pertain to a number of difficulties that were uncovered at follow-up.

3.1.1 Nonunion of fracture in a smoking patient

Figure 23 (a): 2 days post presentation, plaster of Paris immobilisation in a smoking patient

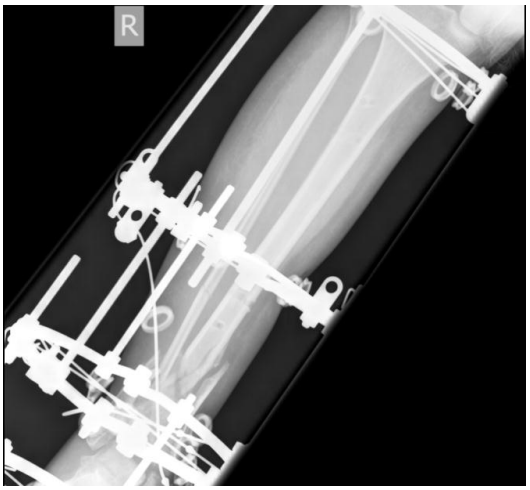


Figure 23 (b): 6 weeks post injury after external fixation (unilateral frame)

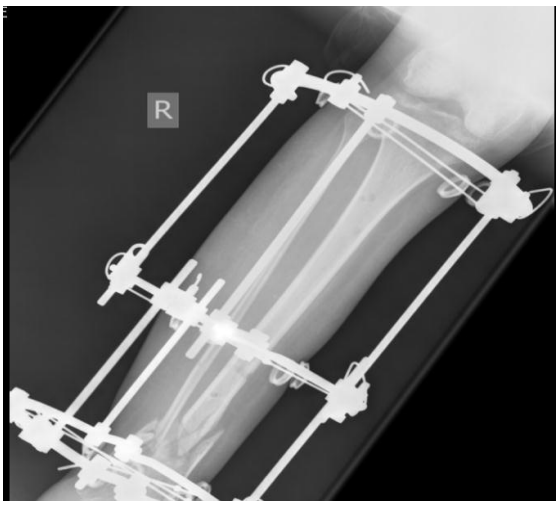


Figure 23 (c): Nonunion of fracture, 6 months post-reduction

Figure 23 (a), (b) and (c): Radiographs taken from a smoking patient at presentation (a), 6 weeks post fracture (b) and at follow-up, 6 months after external fixation of the tibia and fibula. The patient was a female smoker aged 43 at the time of injury, with no stated comorbidity. Clinical reports state that there was no significant callus seen at 6 months, consistent with nonunion. Interestingly, no cells were expressed from this patient's sample during processing in the laboratory.

3.1.2 Malunion Due to Obesity

Figure 24 (a): Obese patient 3 days post-injury, after internal fixation

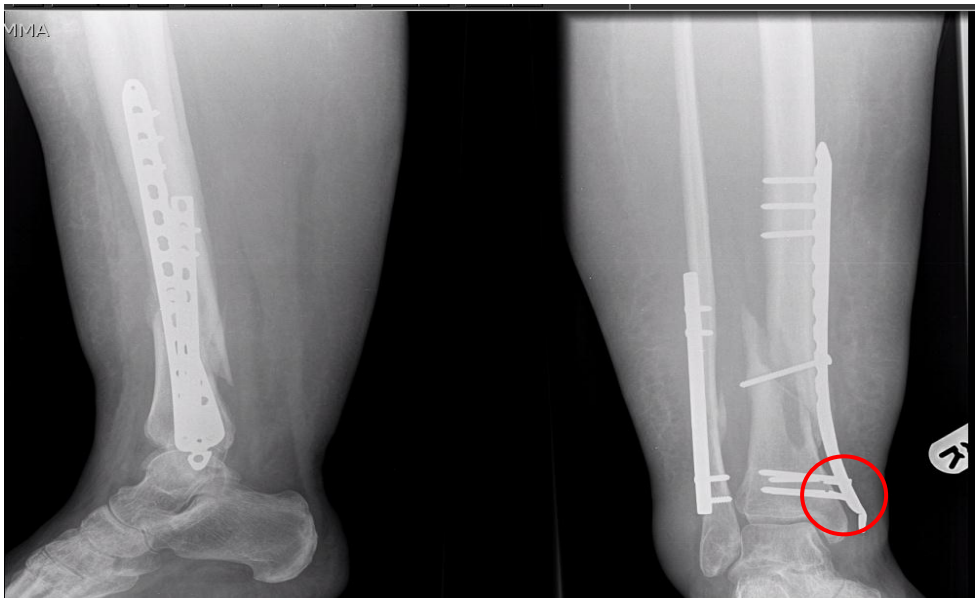


Figure 24 (b): Obese patient 6 months post surgery at follow up

Figure 24 (a) and (b): Radiographs taken from a non-smoking, 33 year old female obese patient at presentation (a) and (b), at follow-up, 6 months after internal operative fixation of the tibia and fibula. The right image on radiograph (b) depicts broken screws at the distal portion of the lateral tibia (red circle).

3.1.3 Patient with Osteopenia

(a) (b)

Figures 25 (a) and (b): Oblique x-ray images of osteopenic patient on day 1 of presentation of fracture

Figure 25 (a), (b) and (c): Radiographs taken from a non-smoking, 84 year old female osteopenic patient at presentation (a) and (b) and at follow-up (c), 6 months after fracture of the tibia and fibula.

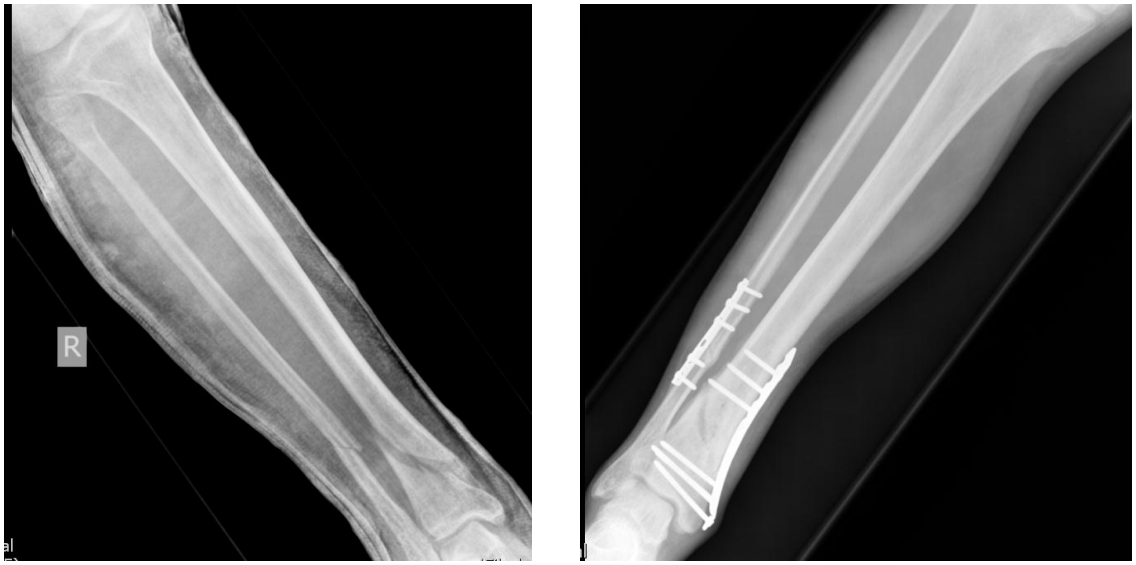
3.1.4 Patient with a History of Compartment Syndrome

(a) (b)

Figures 26 (a) and (b): Images of patient with compartment syndrome on (a) day 1 and (b) 6 months following fracture of tibia and fibula

Figure 26 (a) and (b): Radiographs taken from a non-smoking, 27 year old male patient, with a history of compartment syndrome and prolonged pain (for 6 months) at presentation (a) and (b) at follow-up, 6 months after internal fixation of the tibia.

3.1.5 Patient with Crohn's Disease



(a) (b)

Figures 27 (a) and (b): Patient with Crohn's Disease

Figure 27 (a) and (b): Radiographs taken from a non-smoking, 35 year old male, with a history of Crohn's disease. Radiographs show fracture at presentation (a) and (b) at follow-up, 6 months after internal fixation of the tibia and fibula.

3.2 FRACTURE HAEMATOMA CELL CULTURES

The cellular morphology in the following micrographs (Figures 28-34), is indicative of a progenitor lineage and rapid division and widespread growth was observed in the cultures. Cells were of a fibroblast-like, undifferentiated nature, spindle-shaped morphologically and were able to replicate themselves in the unspecialised culture media. The cells readily became dislodged from the polystyrene substratum of the tissue culture flask by using a solution of trypsin and EDTA during the subculture, rendering them suitable for *in vitro* studies.

Figures 28-34: Fracture Haematoma Cells in Culture

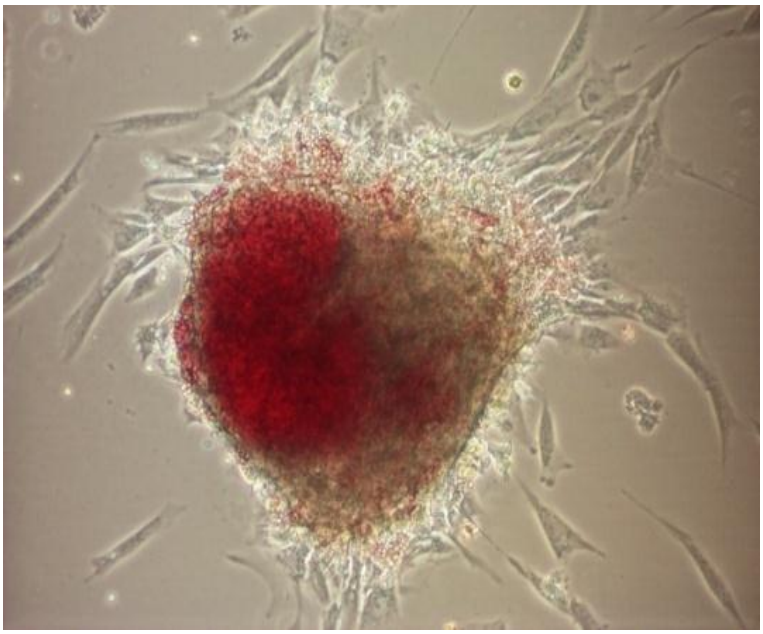


Figure 28: A micrograph (x100) of fibroblast-like cells, migrating from the explanted fracture haematoma *in vitro*, after 7 days in the primary culture following collection from the patient.

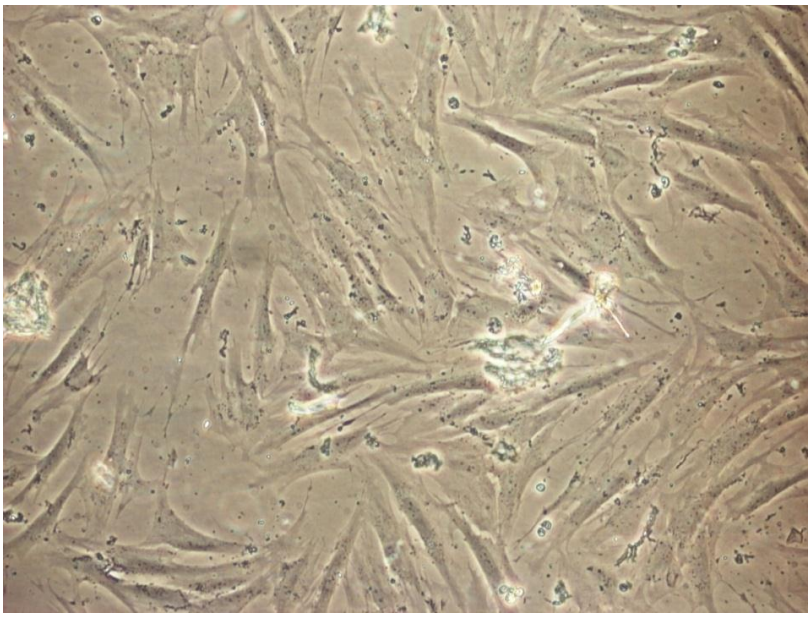


Figure 29: Micrograph of fibroblast-like cells (x100), isolated from the fracture haematoma, after 72 hours in the first passage and displaying rapid growth.

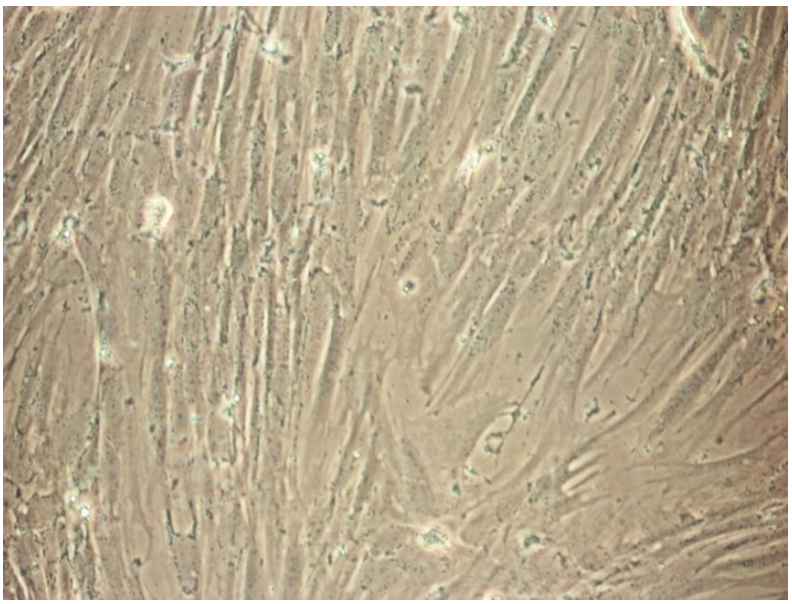


Figure 30: Micrograph of confluent fibroblast-like cells (x100), after 7 days in the first passage.

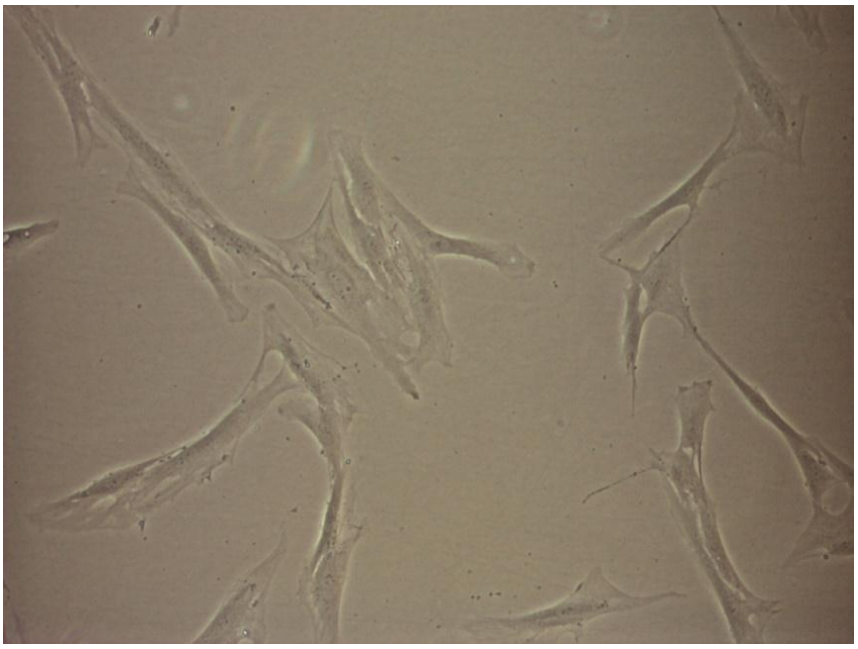


Figure 31: Micrograph of haematoma cells after 48 h in the second passage (x100).

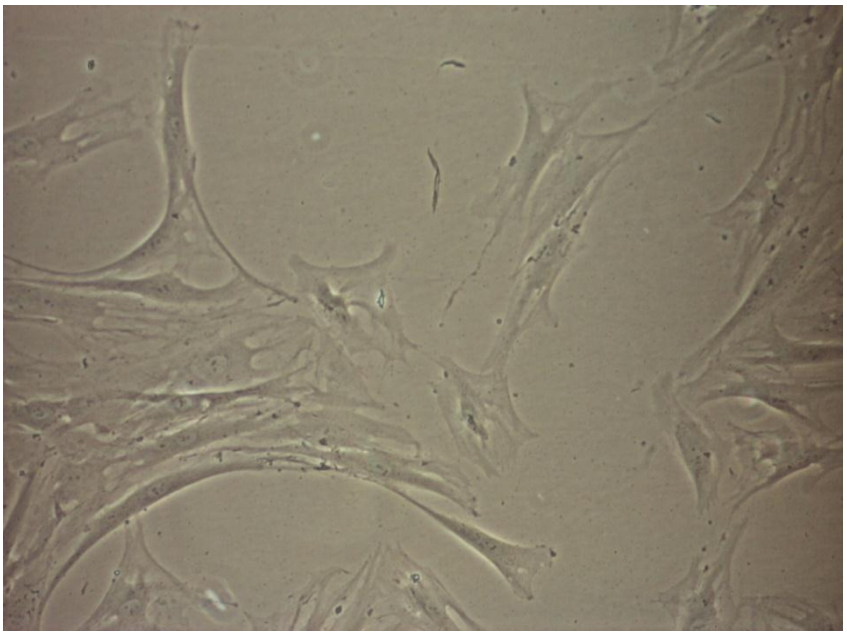


Figure 32: Micrograph of haematoma fibroblast-like cells (x100) after 72h in the second passage.

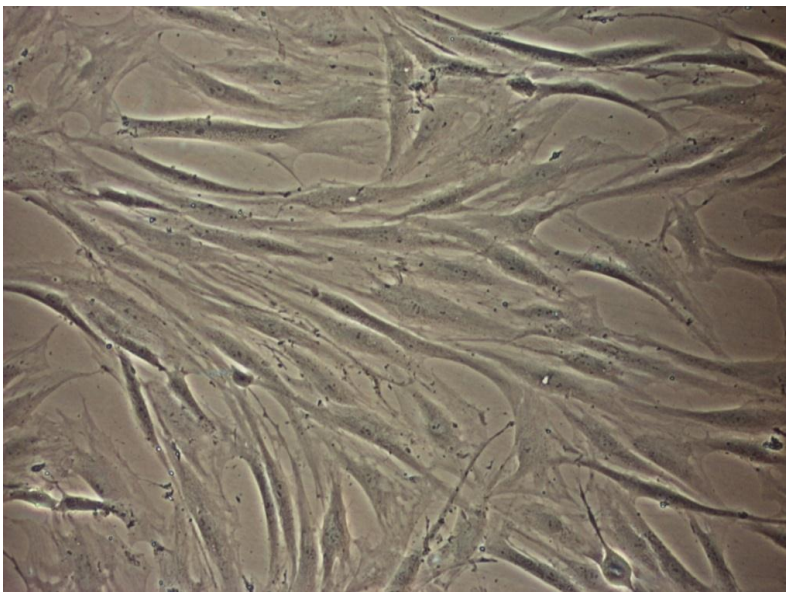


Figure 33: Micrograph of haematoma fibroblast-like cells (x100) in the 7th day of the second passage.

3.2.1 Mesenchymal Stem Cell Control Slide

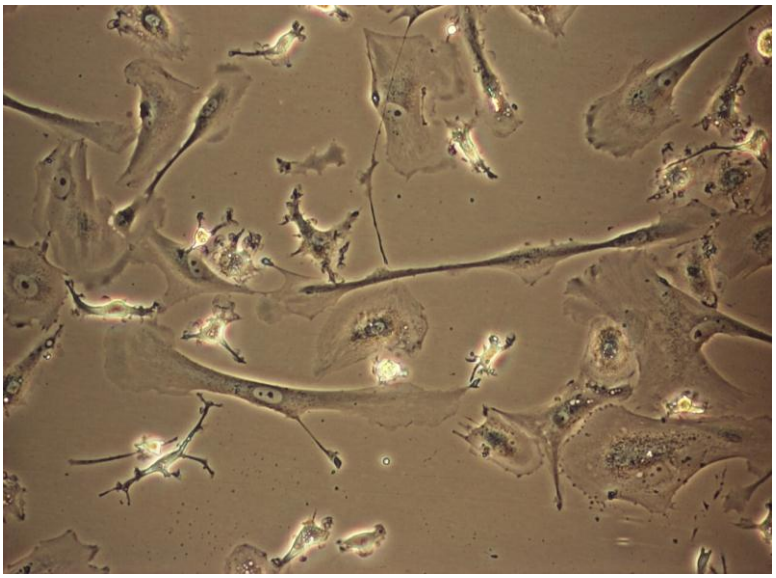


Figure 34: Micrograph of MSCs cultured from control commercial cell line (Lonza, Tokyo).

3.3 CHARACTERISATION OF HAEMATOMA CELLS USING QUALITATIVE IMMUNOCYTOCHEMICAL ANALYSES

3.3.1 Immunofluorescence via Fluorescent Microscopy

The following images were observed using a fluorescent microscope after cyto-spinning and subsequent probing, of the extracted cells with various commercially-available antibodies for characterisation. All cells were taken from 5^{th} passages. The antibody binds to the specific cell surface antigens and fluoresces due to the presence of conjugated FITC on the haematoma cells.

Figure 35: CD29. Immunofluorescent labelling of slide-mounted cells isolated from haematoma cells using antibodies against CD29, a known mesenchymal stem cell surface antigen.

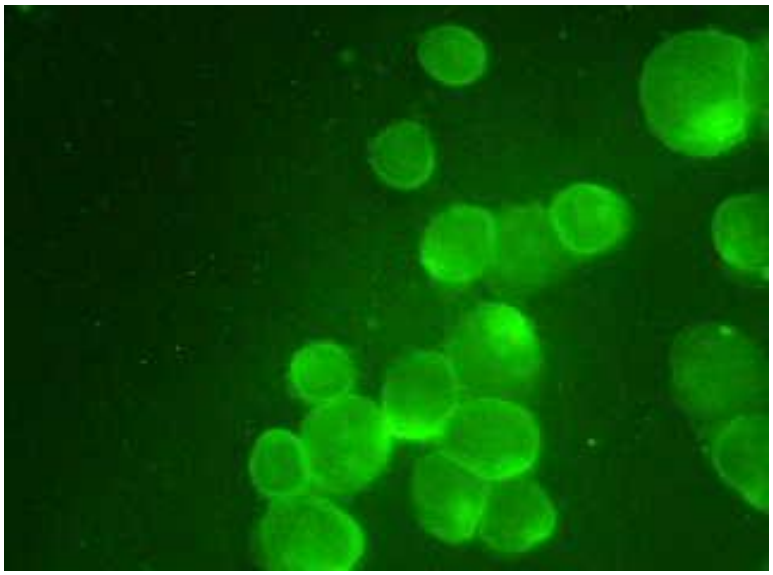


Figure 36: CD44. Positive immunofluorescent labelling of cells isolated from haematoma cells using antibodies against CD44, a mesenchymal stem cell surface antigen.

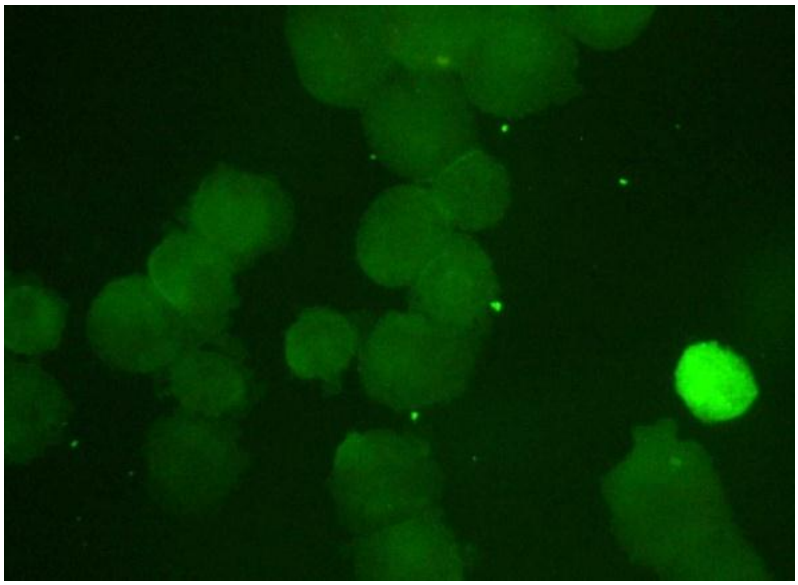


Figure 37: CD105. Immunofluorescent labelling of cells isolated from haematoma cells using antibodies against CD105 (Endoglin), a mesenchymal cell surface antigen.

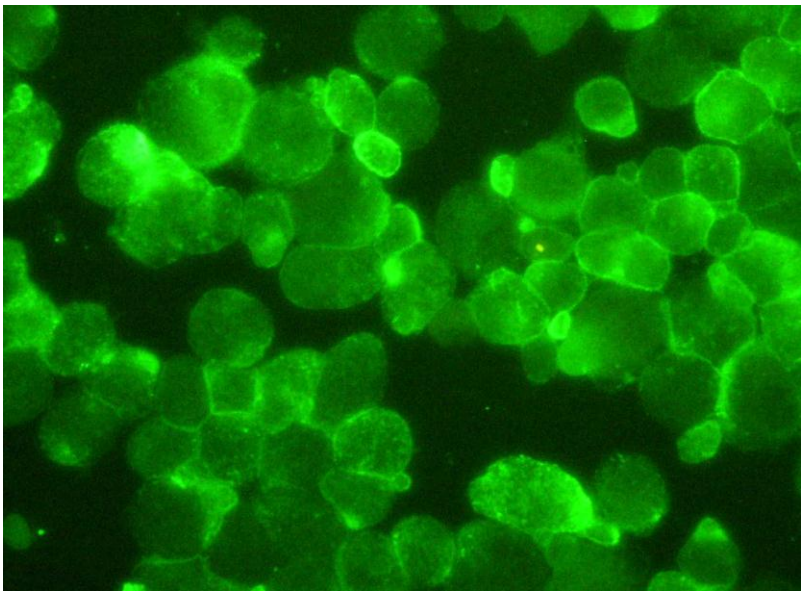


Figure 38: CD166. Immunofluorescent labelling of cells isolated from haematoma cells using antibodies against CD166, a mesenchymal stem cell surface antigen.

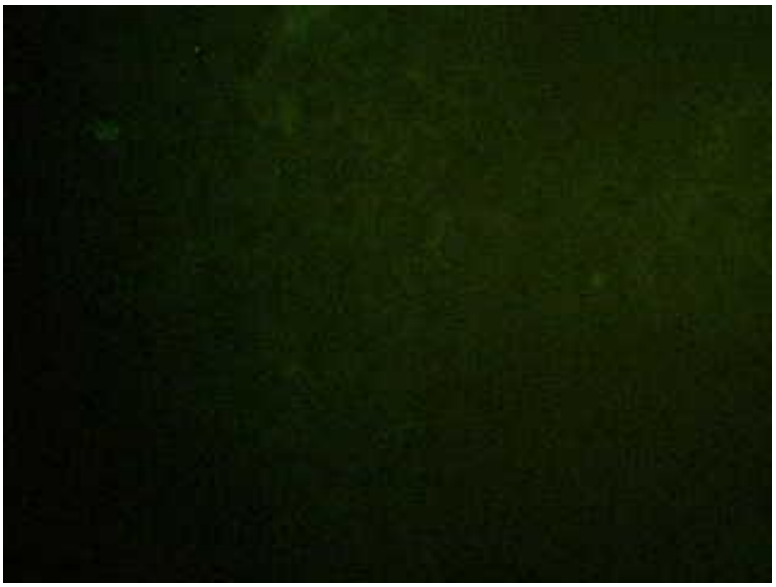


Figure 39: CD34. The haematopoietic stem cell marker, CD34 was used as a negative control on the mesenchymal cells and there was little evidence of uptake of this antibody.

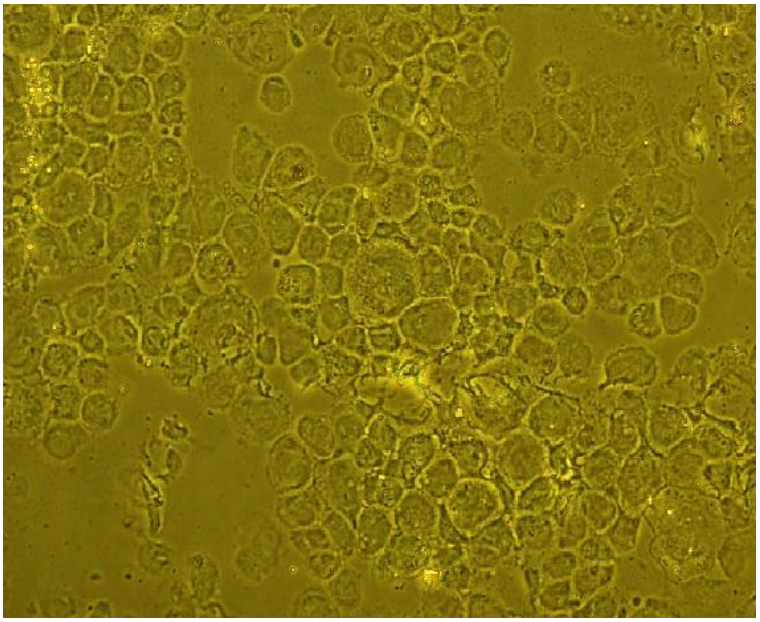


Figure 40: Control Slide. Non-probed MSCs stained with FITC under normal light microscopy

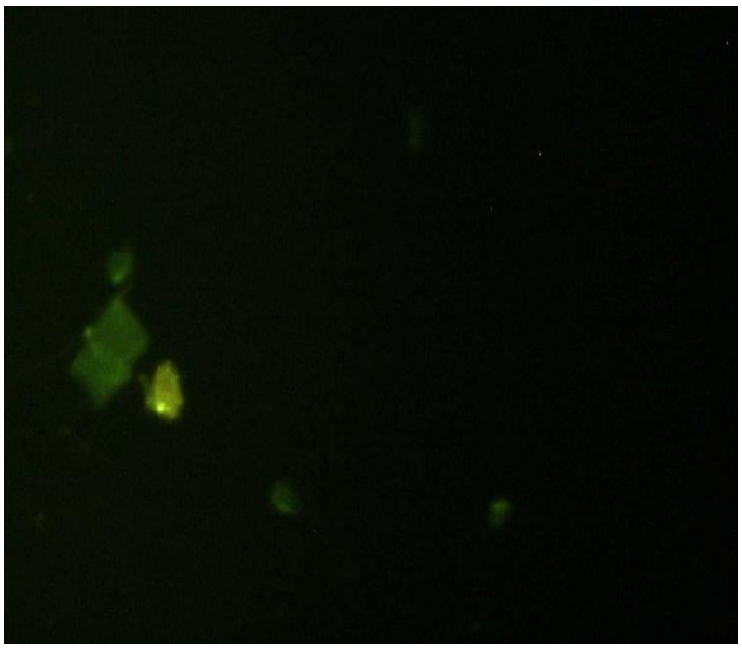


Figure 41: Control Slide. Non-probed MSCs stained with FITC polyclonal antibody under fluorescent microscopy.

3.4 CELL ANALYSES USING FLOW CYTOMETRY

Flow cytometry is a useful tool capable of performing a variety of assays on cell populations. The instrument used in this study (Epics XL; Beckman Coulter, High Wycombe) confirmed the characterisation of the cells extracted from the fracture haematomas, which was done so alongside the immunofluorescence that had previously been performed. *Immunophenotyping* is a quantitative technique that affords 'triangulation' within the study following observation of the typical morphological and physical properties, as well as a series of other immunocytochemical methods and therefore offers improved robustness of the notion that the cells of interest are indeed of a mesenchymal origin. The same CD markers used for the previous fluorescent microscopy assays were used for immunophenotyping; suspended cells were labelled with commercially available antibodies (CD markers), conjugated with either FITC or PE (phycoerythin) as applicable.

3.4.1 Cellular Immunophenotyping via Flow Cytometry

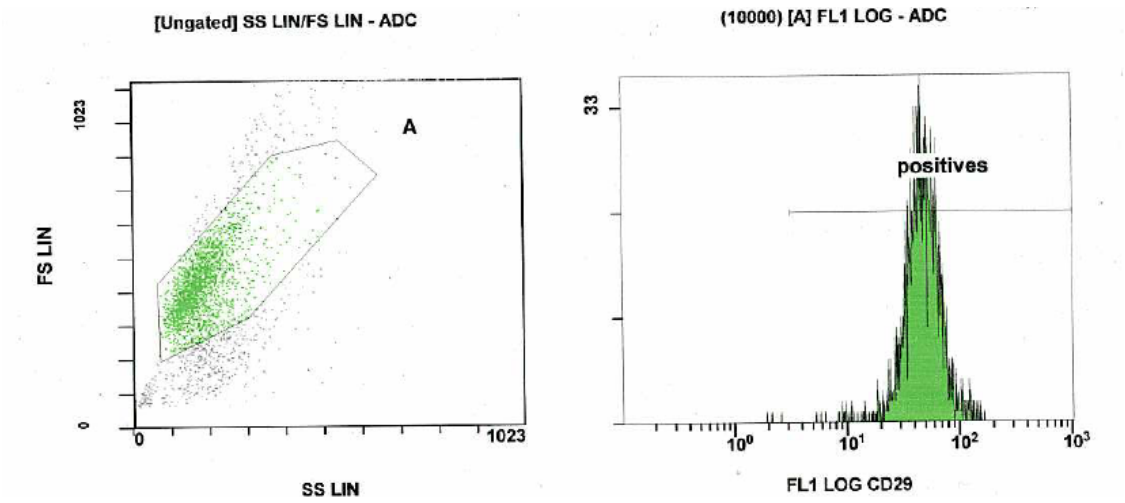


Figure 42: Immunophenotyping CD29. Positive expression of CD29 MSC marker using flow cytometry immunophenotyping.

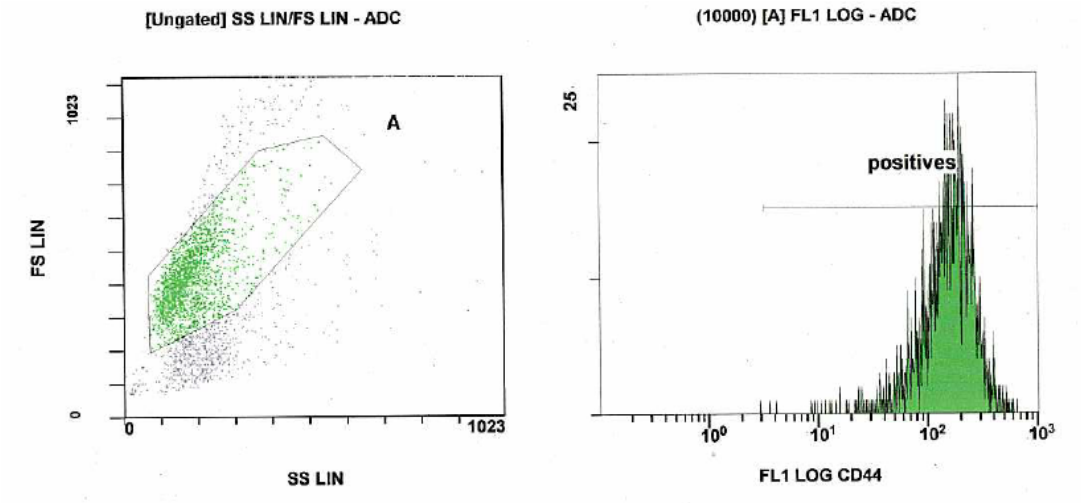


Figure43: Immunophenotyping CD44. Highly-positive expression of CD44 MSC marker via immunophenotyping on flow cytometry.

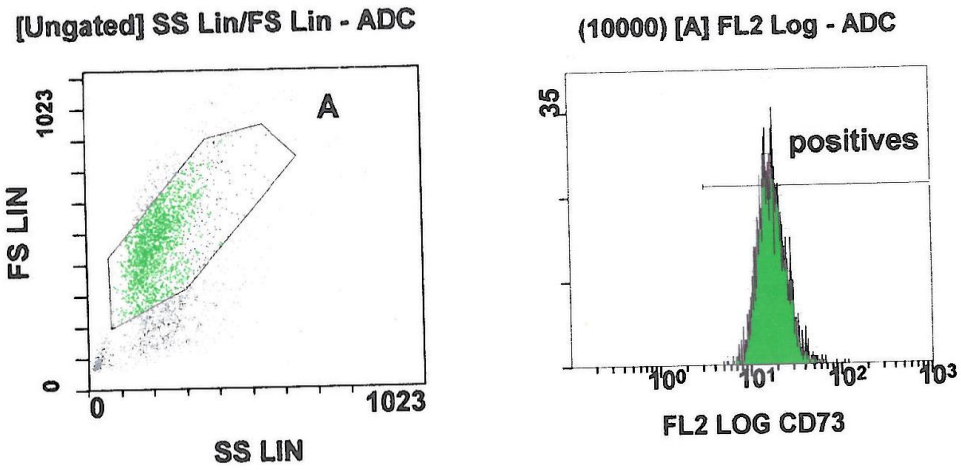


Figure 44: Immunophenotyping CD73. Positive expression of CD73 (PE) MSC marker.

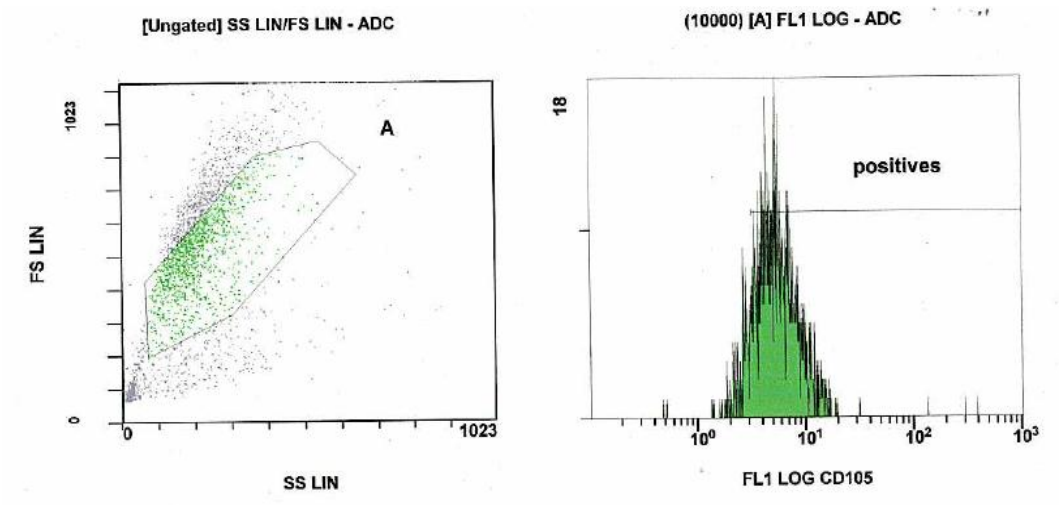


Figure 45: Immunophenotyping CD105. Positive expression of CD105 MSC marker using flow cytometry (FITC).

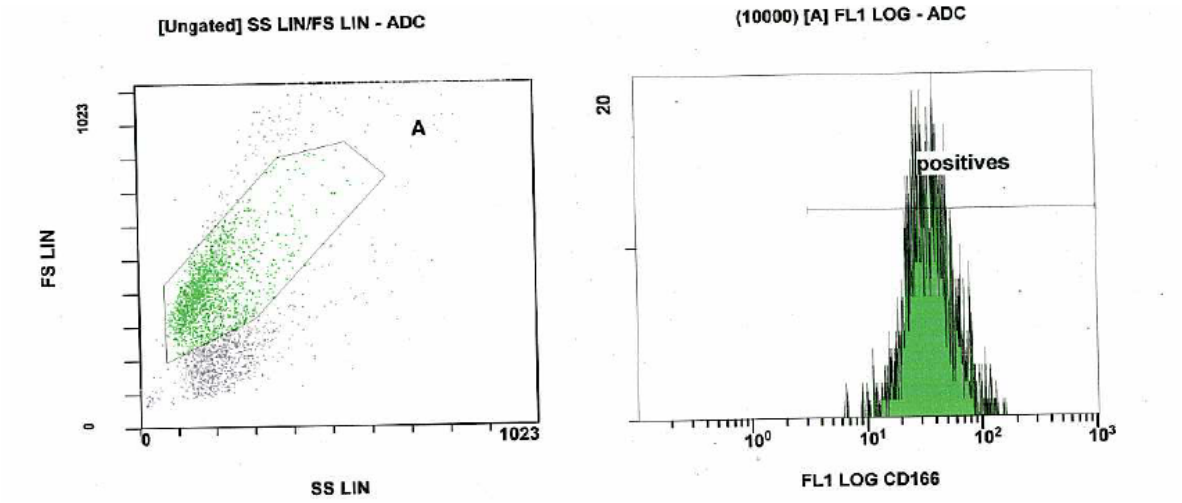


Figure 46: Immunophenotyping CD166. Positive expression of CD166 MSC marker by flow cytometry.

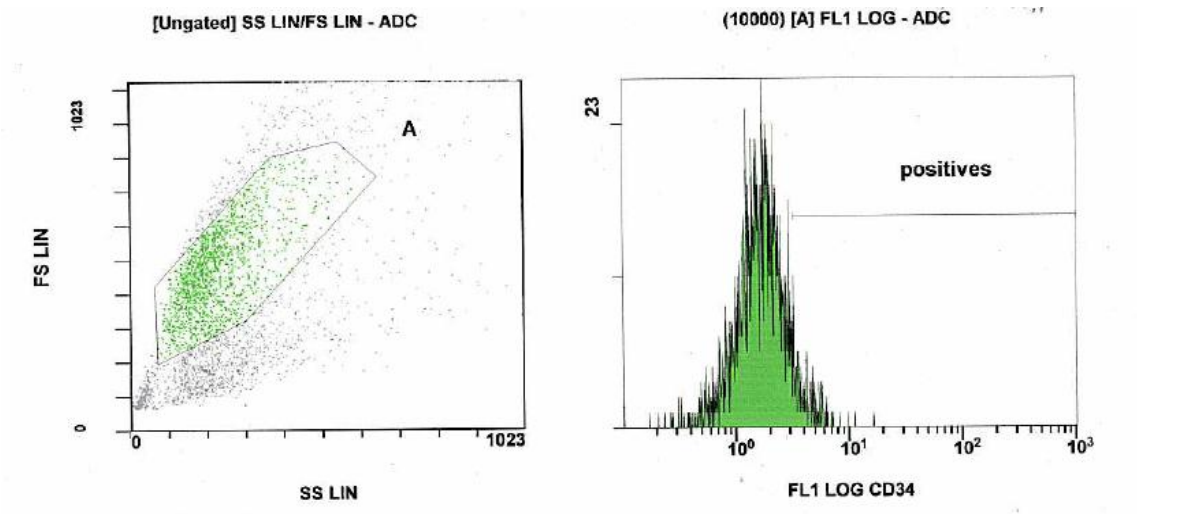


Figure 47: Immunophenotyping CD34. Negative expression of CD34 MSC marker via flow cytometry immunophenotyping, which was used as a negative control.

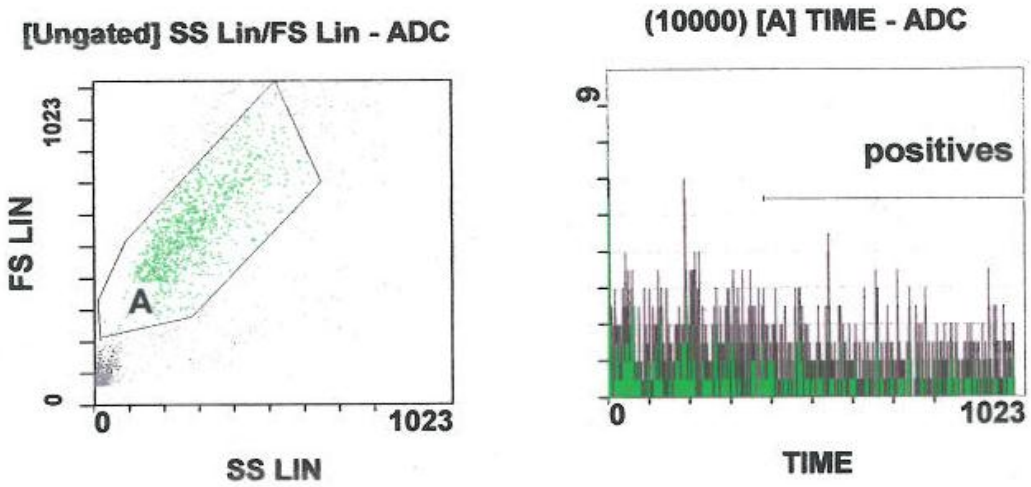


Figure 48: Immunophenotyping Control. Negative control via flow cytometry immunophenotyping, using non-probed, FITC-stained MSCs. The histogram on the right shows a negligible amount of autofluorescence taking place in cell suspensions.

Table 10: Summary of Mesenchymal Stem Cell Markers Over 10 Passages

Marker	1st Passage	2nd Passage	3rd Passage	4th Passage	5th Passage	6th Passage	7th Passage	8th Passage	9th Passage	10th Passage
CD29	99.95%	100%	93.18%	88.13%	98.22%	99.78%	99.80%	99.85%	99.63%	100%
CD44	100%	100%	98.27%	97.29%	99.49%	99.79%	99.83%	100%	99.65%	98.87%
CD73(PE)	100%	100%	97.38%	100%	99.82%	100%	100%	100%	100%	100%
CD105	35.72%	47.58	71.96%	83.21%	90.91%	94.44%	91.75%	92.48%	81.40%	69.43%
CD166	99.36%	97.33%	100%	98.86%	98.33%	100%	99.82%	99.65%	99.49%	100%
CD34	69.39%	34.42%	25.35%	19.29%	5.98%	6.85%	6.45%	5.85%	7.68%	6.62%

Table 10: Expression of various cell surface antigens that are known to be expressed by mesenchymal stem cells. The data is representative of cells extracted from one patient's fracture haematoma. Each passage number (1-10) denotes a 5-day culture.

3.5 Cell Population Counting Using Flow Cytometry (FACS)

Having established the identity of the cells of interest isolated from human fracture haematomas, it was possible to conduct analyses that may find differences in cells between smoking and non-smoking groups. Indeed, flow-cytometric counting can determine culture proliferation rates of the cells of interest isolated from the fracture haematoma and would perhaps give an indication of healing performances between patient groups. Qualitative comparisons can be made between smokers and non-smokers, the hypothesis being that cells extracted from smokers are of inferior quality and are disadvantaged in terms of proliferation, growth and development. Quantitative data obtained from flow cytometry can provide information regarding the quantity, volume and granulation of cells processed on a flow cytometer and this preliminary data is shown later.

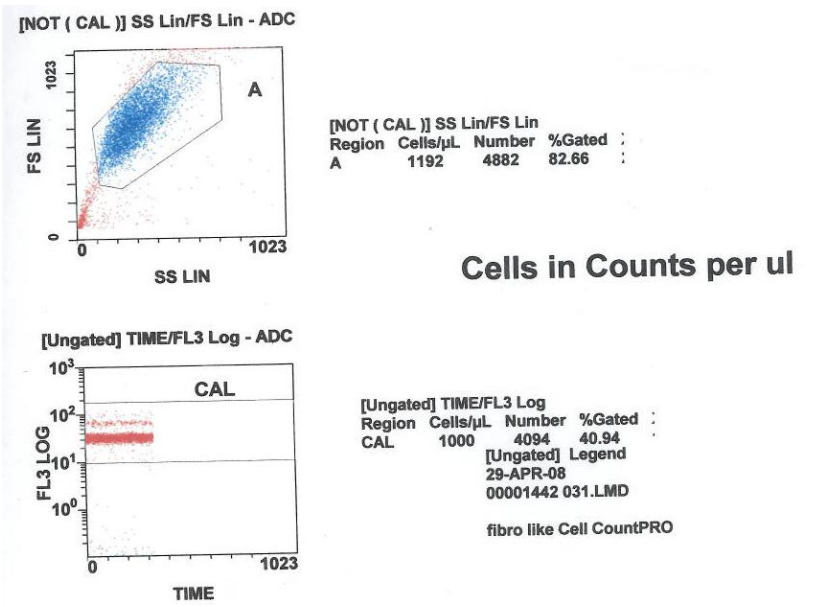


Figure 49: Example of data acquisition from flow cytometer counting using fluorospheres as the calibration medium. The count for this particular sample was 1192 cells per µl.

3.5.1 Flow Cytometry Cell Gating

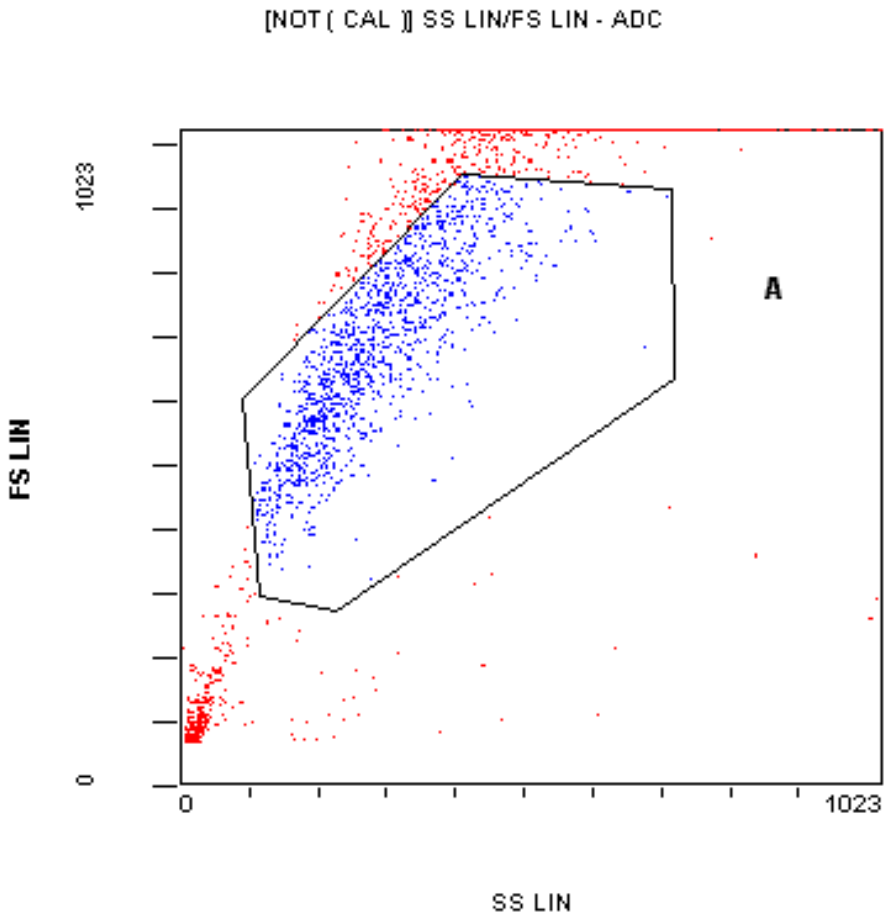
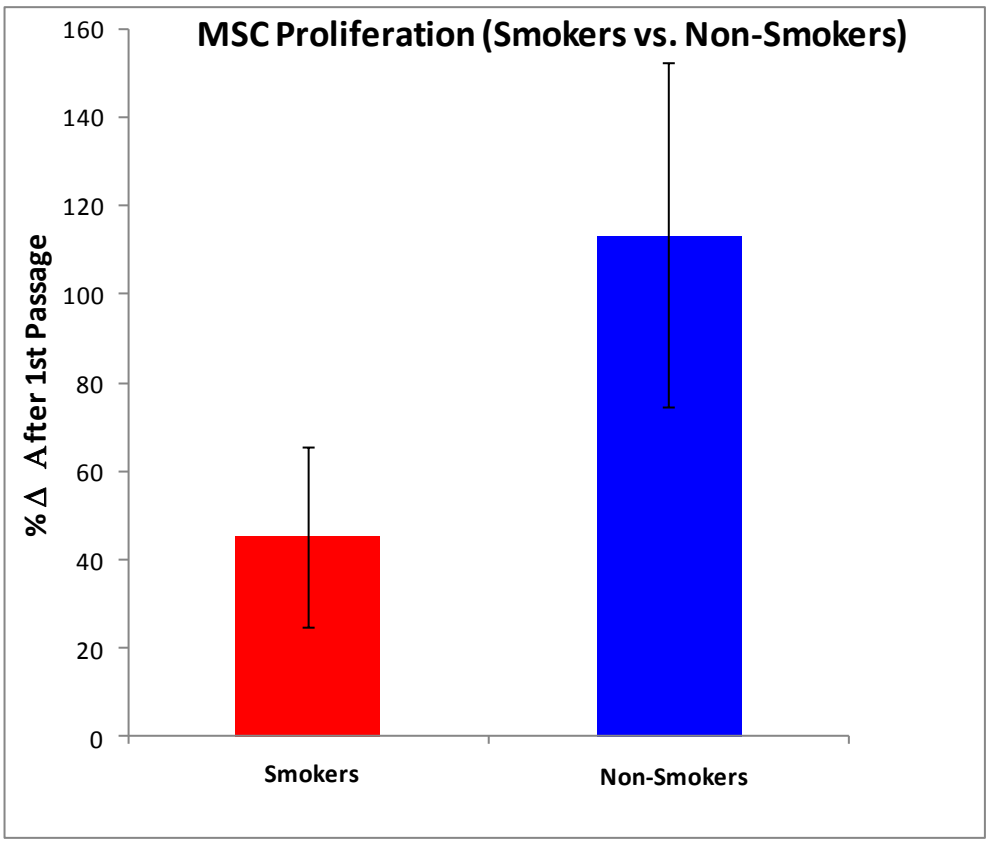


Figure 50: Flow Cytometry Gating

Example of a flow-cytometric plot of fibroblast-like cells taken from a population of cells that had been cultured to a level of confluence *in vitro*. The flow-cytometer is capable of quantitating cells in a given sample (data shown later) and is able to determine this number via the addition of an equal quantity of flow-count Fluorosphere Beads (Beckman-Coulter, High Wycombe). The region of fibroblast-like cells (blue dots) is strategically 'gated', such that particulate debris and obvious doublets (shown as red dots) are precluded from the count for each sample. Actual plots can be found in the appendices.

3.6 ABSOLUTE CELL COUNTING (FACS)

3.6.1 Mesenchymal Stem Cell Proliferation (Smokers vs. Non-Smokers)

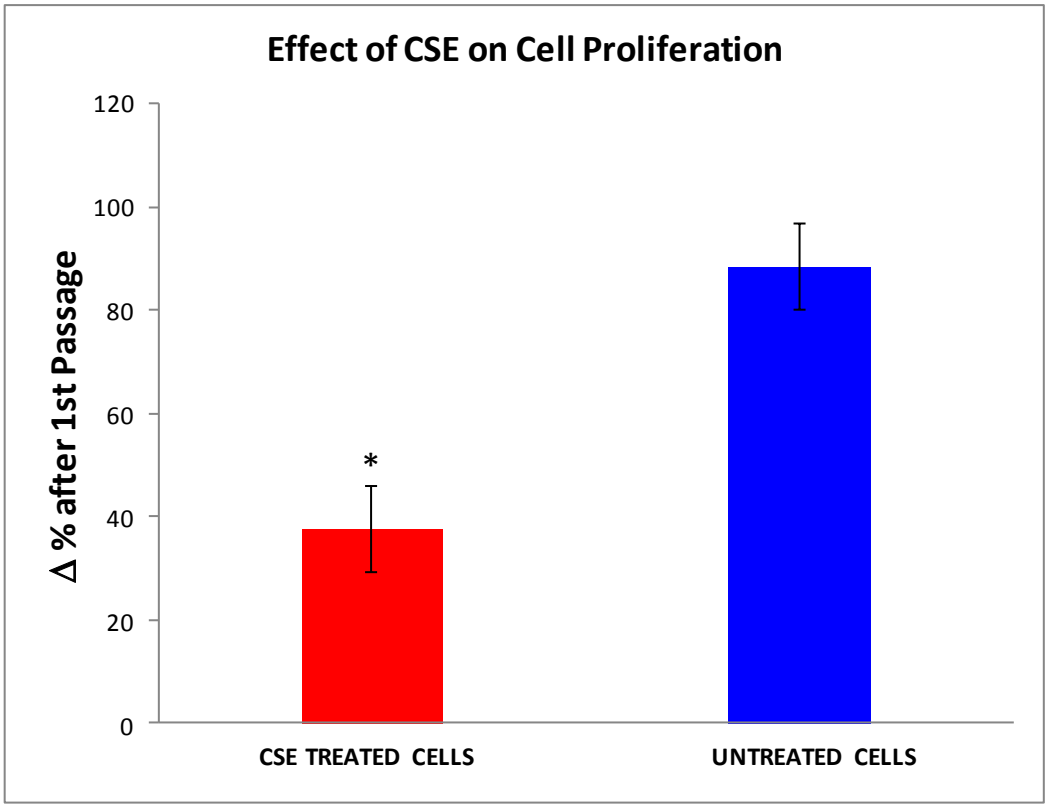


Data not significant (p>0.05; Mann-Whitney U test)

Figure 51: Cell Proliferation

Graph showing cell proliferation differences in fracture patients who were smokers (*n*=7) compared to those who confirmed a non-smoking status (*n*=11). Values are means with standard error bars (±SEM) and represent 5 days of *in vitro* expansion after baseline counts using flow cytometry (%Δ) (actual p=0.17). All cells were taken from 5th passage.

3.6.2 Mesenchymal Stem Cell Proliferation (CSE-Treated vs. Untreated)

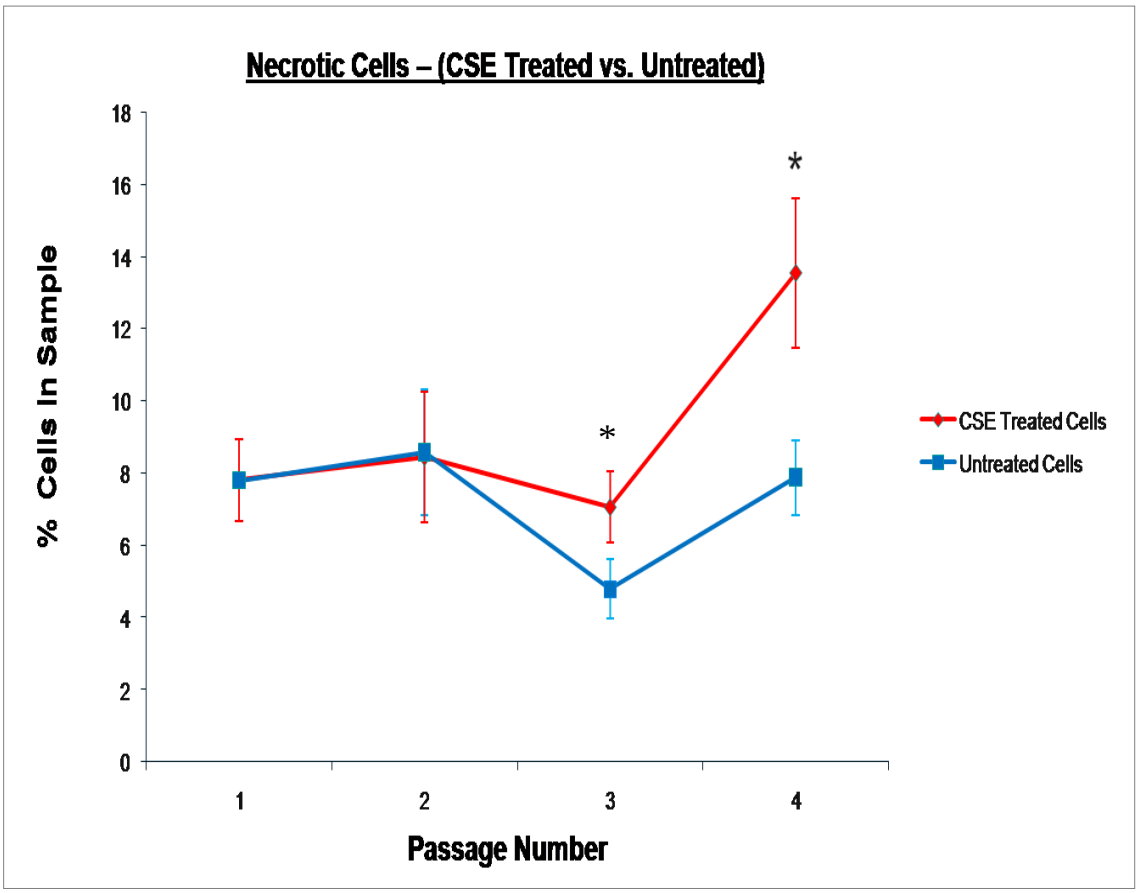


(*p<0.05; Student's *t*-test)

Figure 52: Effect of CSE on Proliferation

Cells extracted from only non-smoking patients (*n*=10) were divided into two groups; one group was treated with CSE and the other left untreated. It can be seen that *in vitro* proliferation rates in those cells infused with CSE were significantly reduced when quantitated after five days (d5) when compared to the untreated group using ANOVA. Values are mean counts, normalised into % change (% Δ) from baseline after 5d; standard error bars (±SEM) are also shown. All cells were taken from 5th passage.

3.6.3 Cell Necrosis Comparison (CSE-Treated vs. Untreated)

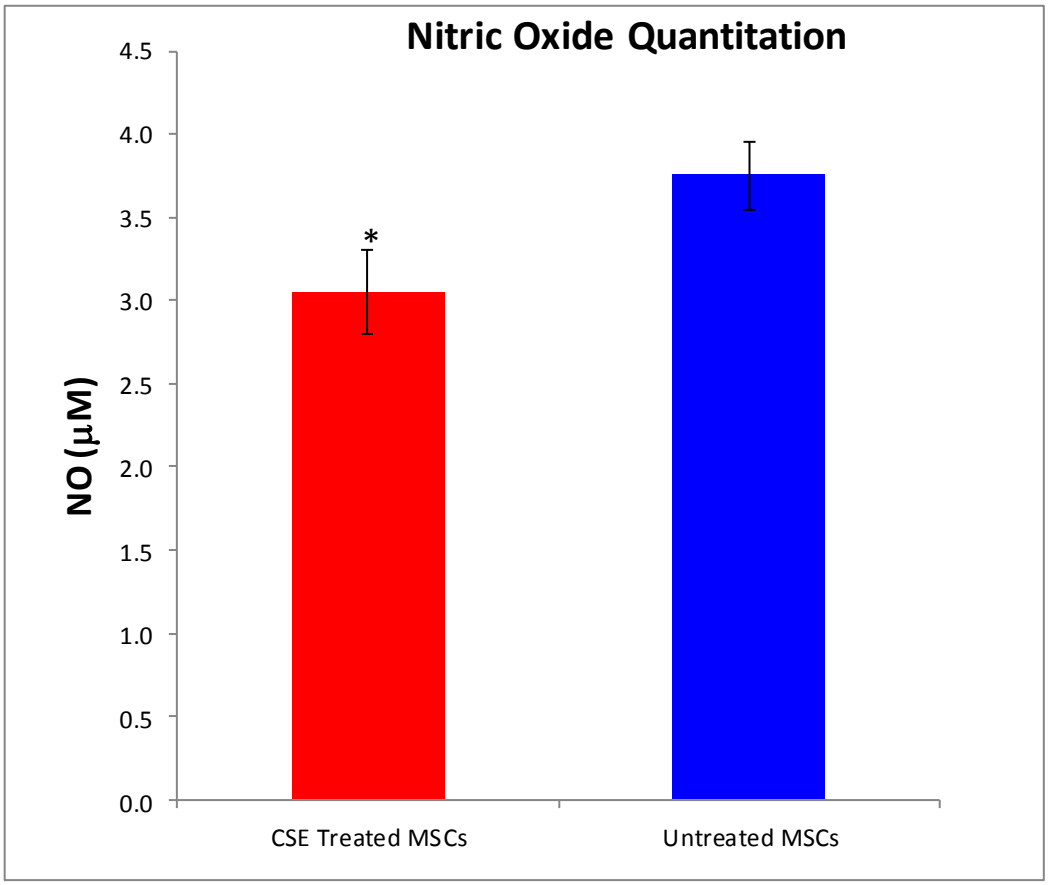


(*p<0.05; Student's *t*-test)

Figure 53: Effect of CSE on Necrosis

Graph showing necrosis in cell cultures (CSE-treated vs. untreated) as detected via propidium iodide flow cytometry; values are mean with standard error bars (±SEM). After culturing through 4 passages, non-smoker cells treated with CSE (*n*=10) had a higher proportion of necrotic cells than the untreated group. There was a further increase of necrosis in the subsequent passage in CSE cultures.

3.6.4 Nitric Oxide Levels in Lysed Cells (CSE Treated vs. Untreated)



(*p=0.05; Student's *t*-test)

Figure 54: Effect of CSE on Nitric Oxide

Graph to show cytoplasmic nitric oxide levels in CSE-treated vs. untreated lysed cell cultures; values are means with standard error bars (±SEM). The CSE-treated group (*n*=10) showed reduced levels (p=0.05) of nitric oxide within the cytoplasm of the haematoma-derived mesenchymal stem cells when compared to the untreated group (*n*=10). All cells extracted for the assay were extracted from non-smoking patients.

3.6.5 Recovery of MSCs Subjected to CSE Infusion

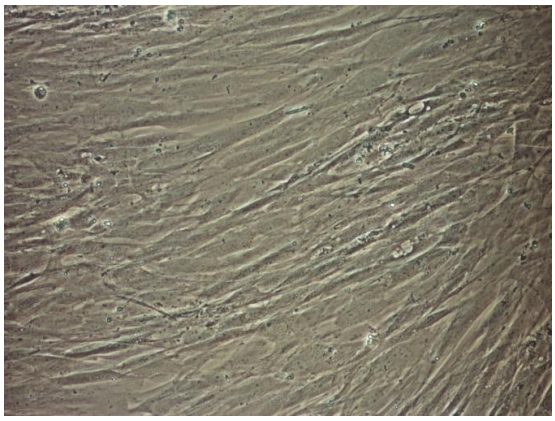


Figure 55 a) Cells before CSE Infusion (d7 of culture)

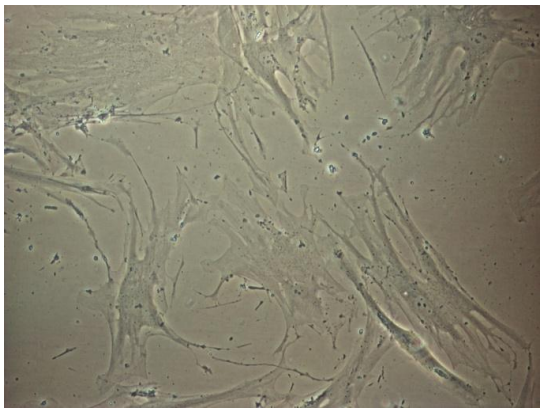


Figure 55 b) Cells after CSE Infusion (2.5%) (d21)

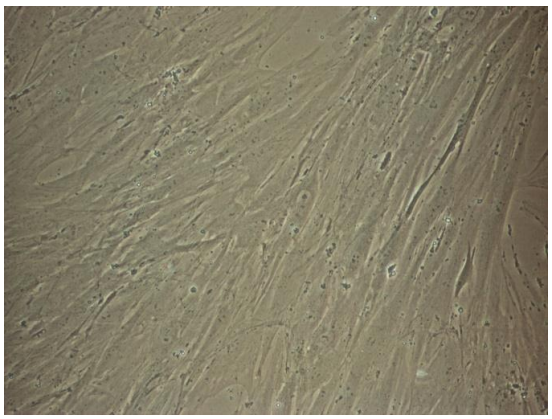
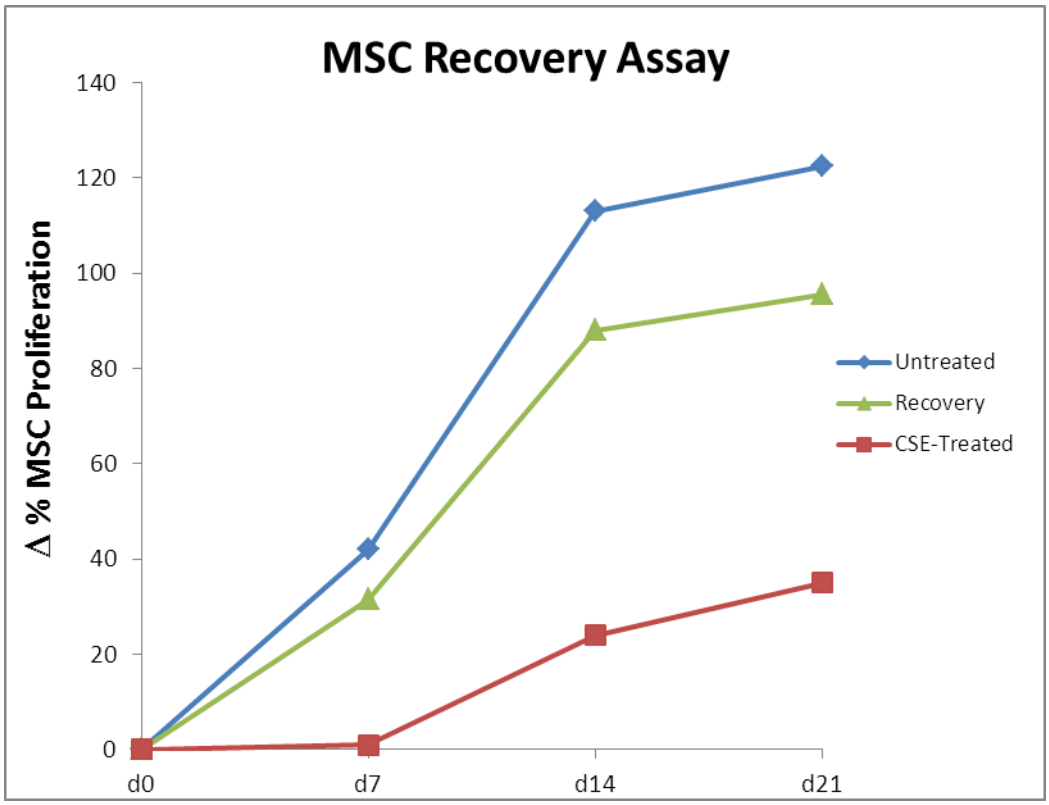


Figure 55 c) Cell recovery after withdrawal of CSE (d35)

Figures 55 a) – c) (Previous Page): a) Cells were cultured in IMDM and allowed to become confluent. After 7 days, CSE (2.5%; 20 cigarettes/day equiv.) was infused into the cell culture media. b) After 14 days of CSE infusion (d21), cell cultures became less concentrated and lamellipodia structures in cells became thinned and fragmented. c) Cultures were then allowed to recover following infusions in untreated IMDM for 14 days. Cells were observed, using light microscopy, to be recovering in the absence of CSE; populations increased and regained a notable state of confluence.

3.6.6 MSC Cell Recovery Assay

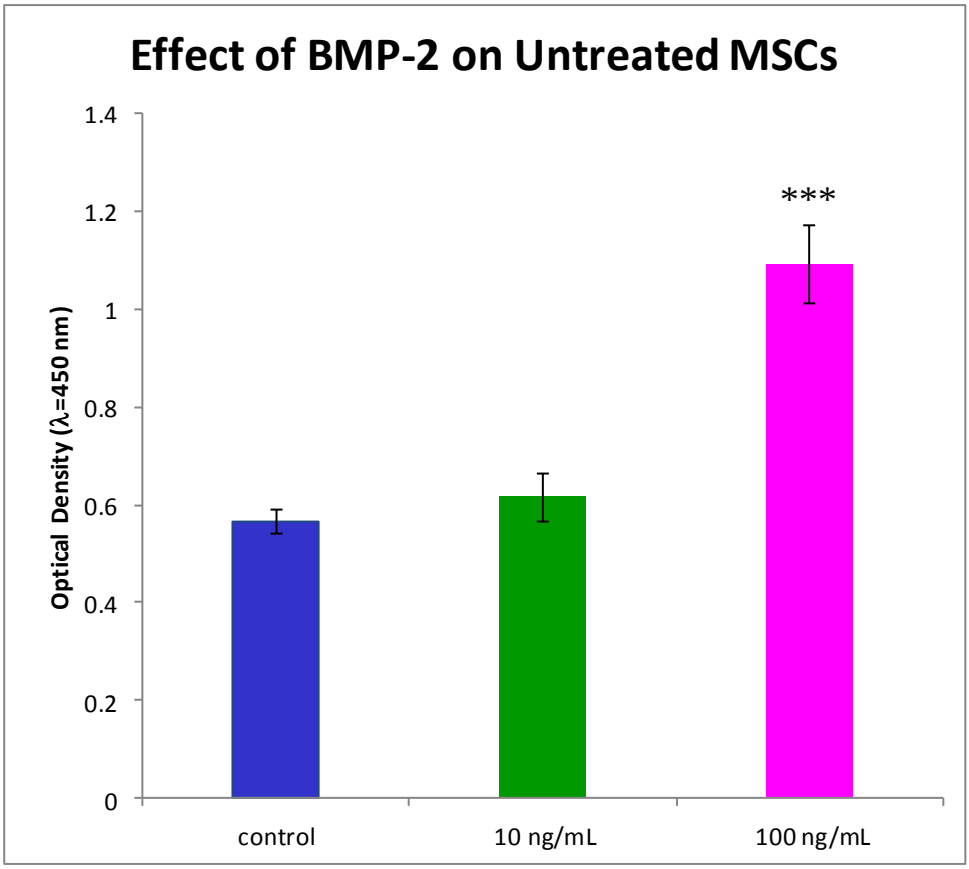


Data not significant ($p>0.05$; ANOVA)

Figure 56: MSC Recovery Assay

Graph showing haematoma cell recovery following withdrawal of a 5-day infusion of CSE from cell cultures after 7, 14 and 21 days (n=10), against untreated (n=10) and continuous (n=10) CSE infusion. All values are normalised from the mean proliferation data in the respective groups. Error bars not shown due to high levels of variance within analyses.

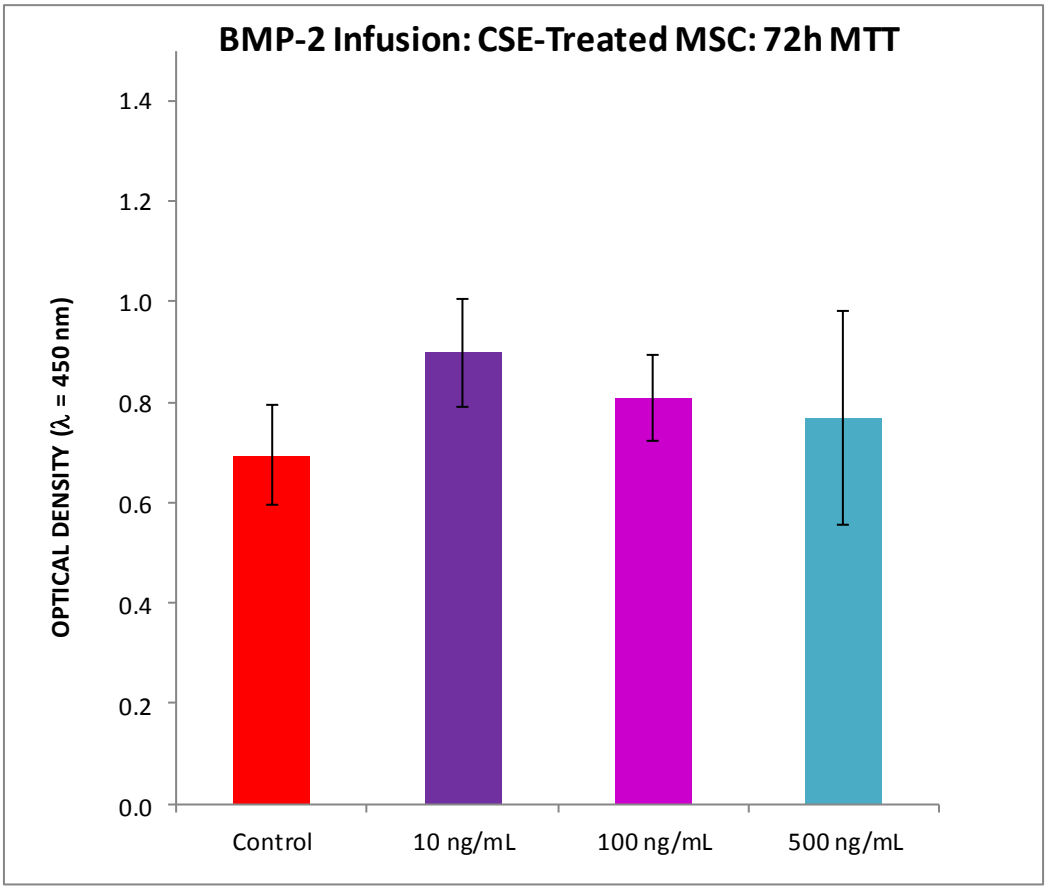
3.6.7 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay: In Vitro Effect of BMP-2 on Cell Viability Enhancement



(***P <0.0001; ANOVA)

Figure 57: BMP-2 Infusion in MSCS in Normal Environment.

In vitro effects on MSC development after 72h of various amounts (10 and 100 ng mL^{-1}) of infused exogenous bone morphogenetic factor-2 (BMP-2) on MSCs (*n*=10), vs. the control group (BMP-2=0 ng mL^{-1}) (*n*=10), via the MTT assay. Graph shows a concentration of 100 ng mL^{-1} to be highly significant with regard to enhancing cell viability in a normal environment. Values are means with standard error bars (±SEM).



(*p>0.05, data not significant; ANOVA)

Figure 58. BMP-2 Infusion in CSE Environment.

In vitro effects on MSC development after 72h of various amounts (10, 100 and 500 ng mL^{-1} of infused exogenous bone morphogenetic factor-2 (BMP-2), vs. control group (BMP-2=0 ng mL^{-1}), via the MTT assay. All values are mean with standard error bars (±SEM). All cell groups (each *n*=10) were first treated with CSE (2.5%) for 5d prior to BMP-2 infusions.

3.7 MOLECULAR STUDIES

3.7.1 SDS-PAGE – Fracture Haematoma Serum

Following the electrophoresis of haematoma serum, polyacrylamide gels were digitally photographed for documentation purposes and are shown here. In order to ascertain the optimal resolution of protein on the gels, two different amounts of lysates were used; Figure 59 shows an image of a gel where 10μL of loaded sample was applied to each lane. The purposely-overloaded silver nitrate stained gel (see Figure 60) showed no additional bands than those gels stained using the Coomassie blue technique.

The aims of the electrophoresis were to attempt the resolution of acute phase proteins that are known to be present in the haematoma serum. The patient samples were resolved alongside the controls foetal calf serum and human sera derived from peripheral blood and a protein marker (SERVA, Heidelberg) was included to estimate molecular weights of resolved bands. The visible proteins seen only in the haematoma lanes that were resolved at 21kDa and 14 kDa were haptoglobin and haemoglobin chains respectively and this was confirmed using mass spectrometry.

3.7.2 SDS-PAGE of Fracture Haematoma Serum: Coomassie Blue Stain

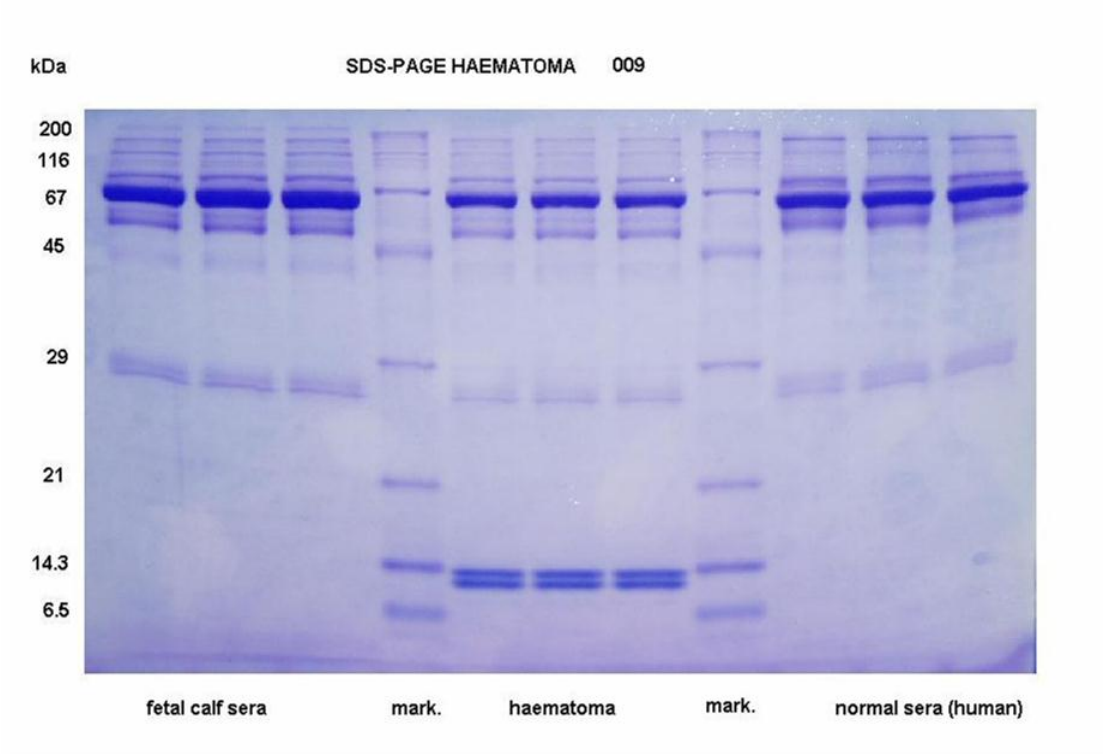


Figure 59: Electrophoresis of Haematoma Serum Proteins (Coomassie Blue).

SDS-PAGE gel (16% polyacrylamide) showing resolved haematoma serum proteins (lanes are haematoma serum; protein marker; foetal calf serum; human peripheral blood serum); lysates were loaded at a rate of 5 μL per well to optimise resolution of bands. This amount equated to approximately 0.01 mg of serum protein in each well, as per published data (Betgovargez *et al.*, 2005). The bands that were resolved at ~67 kDa are serum albumin; the two bands visible at ~14 kDa are haemoglobin α (lower) and haemoglobin β (upper) as identified using mass spectrometry (see later) and represent one patient sample.

3.7.3 SDS-PAGE of Fracture Haematoma: Silver Nitrate Stain

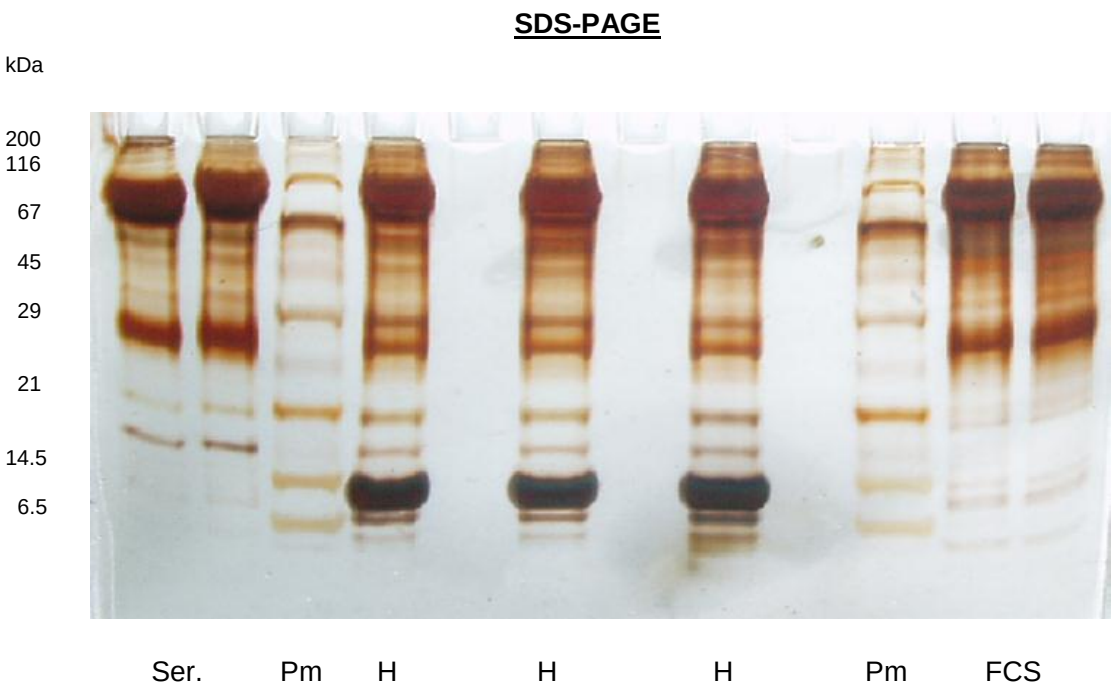


Figure 60. Electrophoresis of Haematoma Serum Proteins (Silver Nitrate).

Photograph of SDS-PAGE (16% polyacrylamide) gel (SERVA, Heidelberg) of fracture haematoma serum proteins (Lanes: Ser.=human serum; Pm = Protein marker; H=Haematoma; FCS=Foetal Calf Serum). The lysates were purposely overloaded at a quantity of 10 μL per well, equating to ~0.02 μg of serum protein in each lane, as again described by Betgovargez *et al.* (2005). A silver nitrate stain was used in an attempt to further improve resolution of the proteins so that relevant acute phase proteins may be isolated from haematoma samples.

3.7.4 Peptide Mass Fingerprint (PMF) for Haemoglobin Alpha (Hb α)

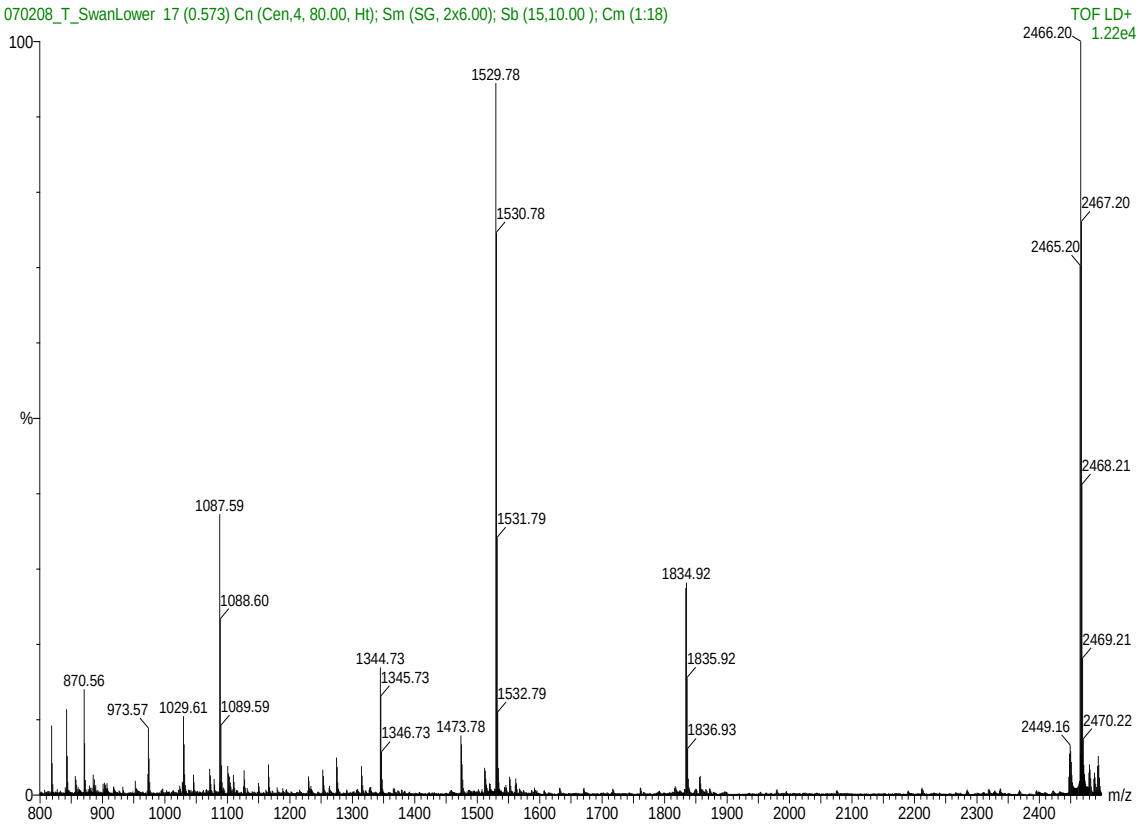


Figure 61: Mass Spectrometry (Hb α).

Peptide Mass Fingerprint (PMF) via mass spectrometry determining the presence of Hb α on the SDS-PAGE gel of the bands seen at the 12kDa level, as derived from serum obtained from the fracture haematoma (courtesy of Kevin Bailey, University of Nottingham).

3.7.5 Peptide Mass Fingerprint (PMF) for Haemoglobin Beta (Hb β)

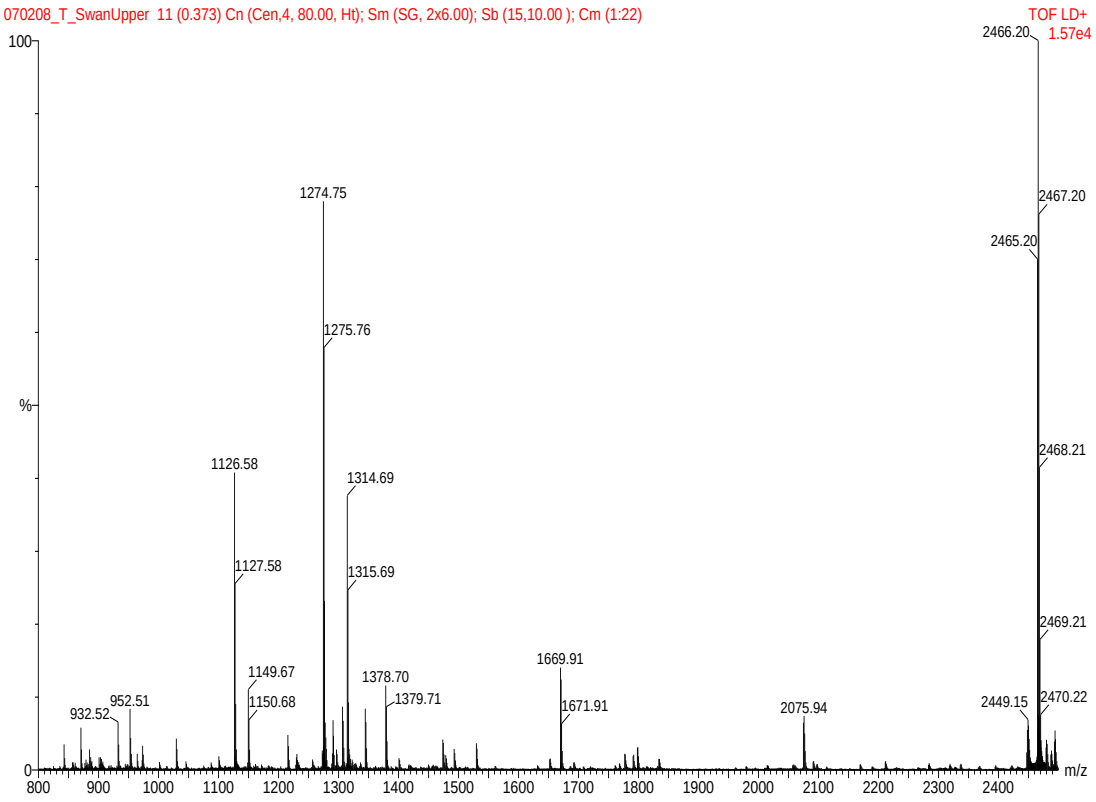


Figure 62: Mass Spectrometry (Hb β).

Peptide Mass Fingerprint (PMF), via mass spectrometry, determining the presence of Hb β on the SDS-PAGE gels, at the 12kDa level, as derived from the serum obtained from fracture haematomas (courtesy of Kevin Bailey, University of Nottingham).

3.7.6 Peptide Mass Fingerprint (PMF) for Haptoglobin (Hp)

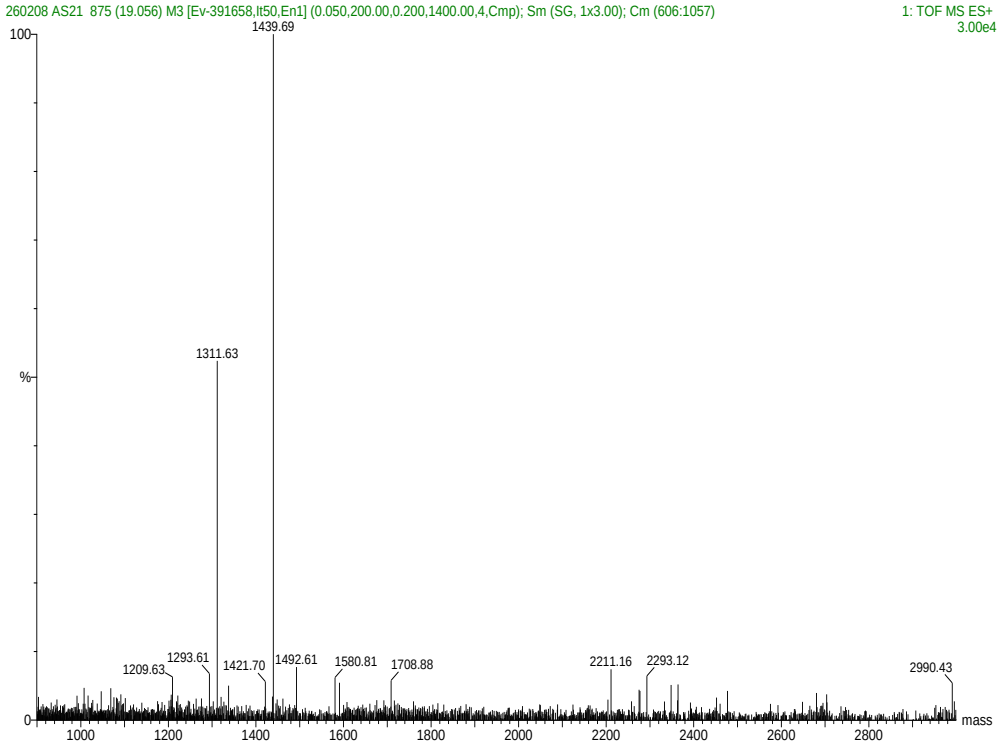


Figure 63: Mass Spectrometry (Hp).

Peptide Mass Fingerprint (PMF), via mass spectrometry, determining the presence of haptoglobin (Hp) on the SDS-PAGE gels (12kDa level) in the serum of fracture haematoma (courtesy of Kevin Bailey, University of Nottingham).

3.7.7 Intracellular Labelling of MSCs Using IL-6 and TGF-β: A Preliminary Study

The following flow cytometry histograms show cytoplasmic expression of the acute-phase proteins IL-6 and TGF-β in MSCs extracted from smoking and non-smoking patients.

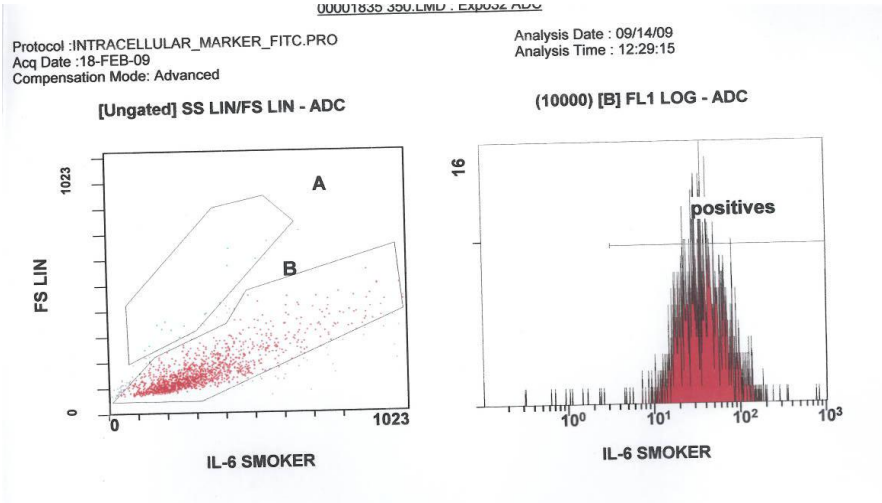


Figure 64. Flow cytometry histogram showing intracellular expression of IL-6 in MSCs extracted from a smoking patient.

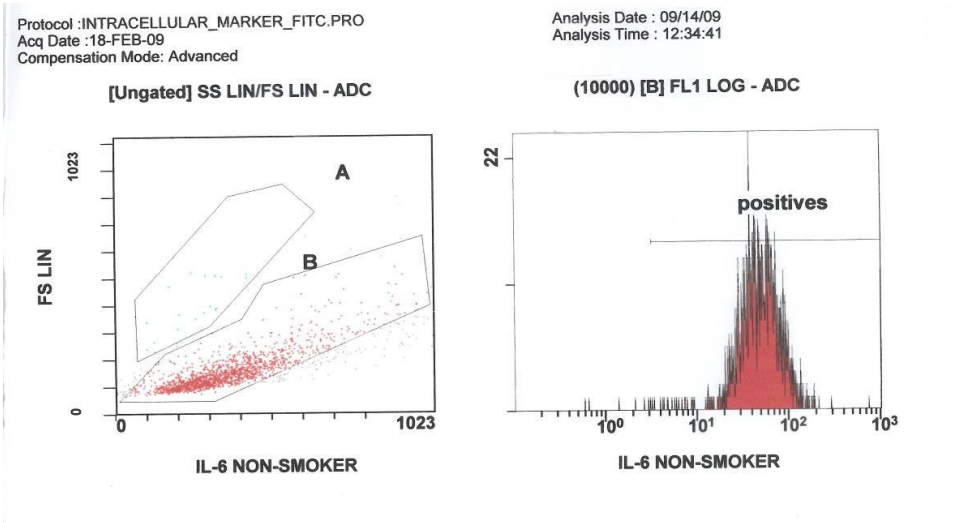


Figure 65. Flow cytometry histogram showing intracellular expression of IL-6 in MSCs extracted from a non-smoking patient.

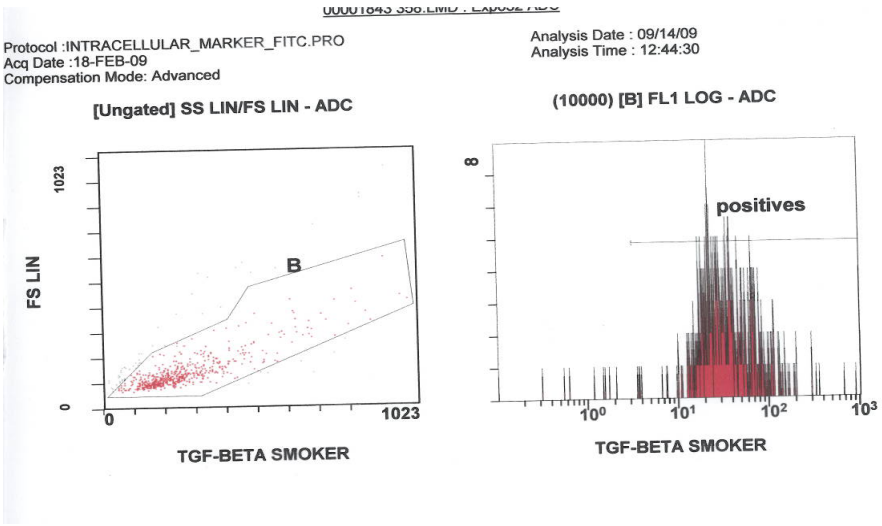


Figure 66. Flow cytometry histogram showing intracellular expression of TGF-β in MSCs extracted from a smoking patient.

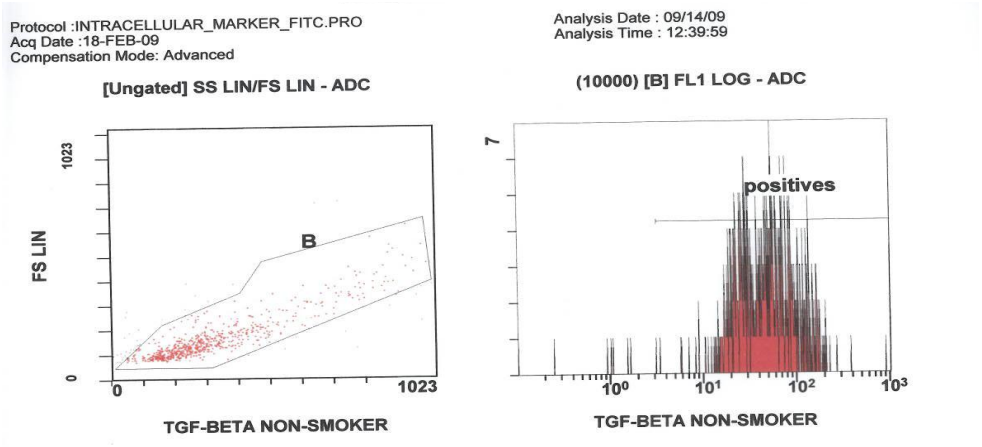


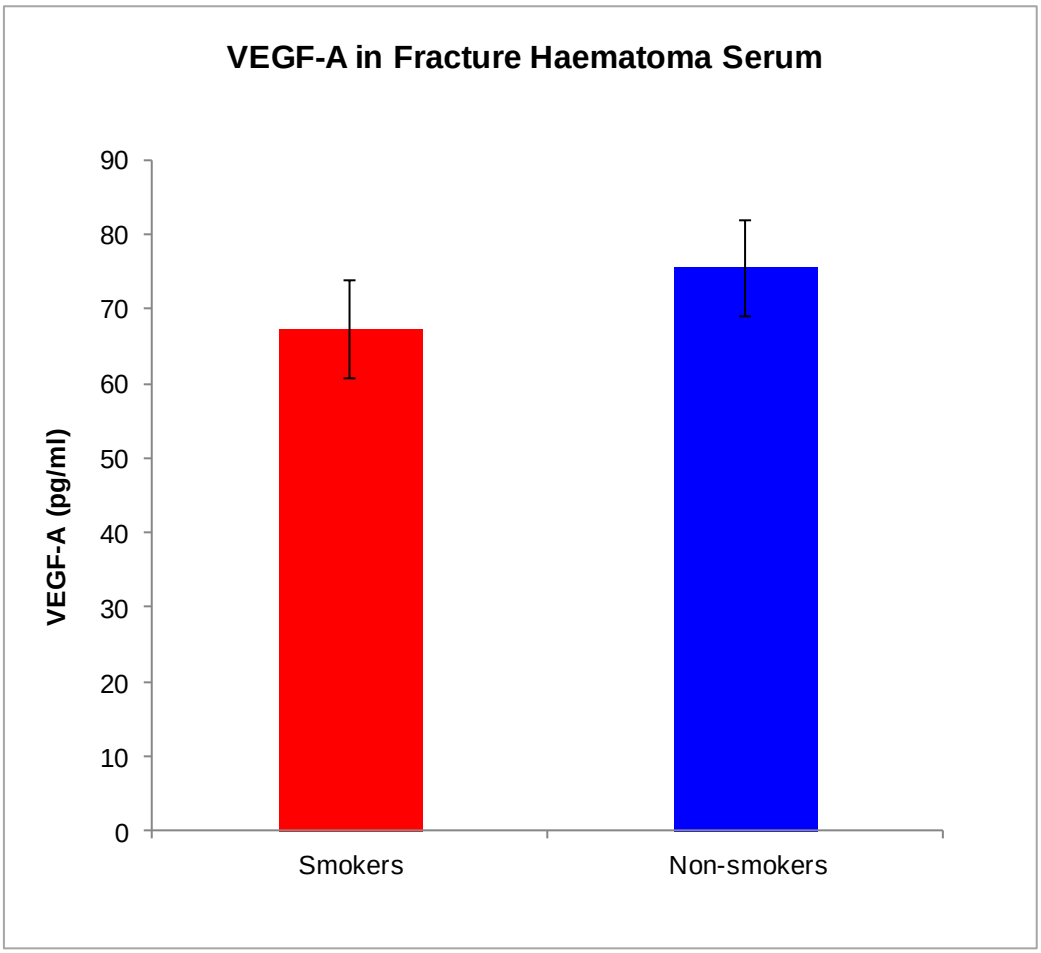
Figure 67. Flow cytometry histogram showing intracellular expression of TGF-β in MSCs extracted from a non-smoking patient.

Sample No.→ **Protein of Interest** ↓	**1**	**2**	**3**	**4**	**Mean** $\bar{(\chi)}$
IL-6 (Smokers)	98.69%	99.05%	99.20%	98.55%	98.87%
IL-6 (Non-Smokers)	98.26%	97.78%	98.52%	99.55%	98.53%
TGF-β (Smokers)	99.23%	97.42%	98.45%	98.82%	98.48%
TGF-β (Non-Smokers)	97.97%	98.90%	98.81%	94.23%	97.48%

Table 11: Intracellular IL-6 and TGF-β

Tabulated summary of preliminary intracellular protein labelling of IL-6 and TGF-β via flow cytometry, preliminary data: MSCs were extracted from non-smokers (n=4) and smokers (n=4). MSC membranes were permeablised prior to probing with IL-6 and TGB-β (separately) and then analysed on a flow cytometer. Values shown are amount of cells positive for each intracellular marker, with the respective mean value in the final column.

3.7.8 Quantitation of VEGF-A via ELISA in Fracture Haematoma Serum (Smokers vs. Non-Smokers): A Preliminary Study

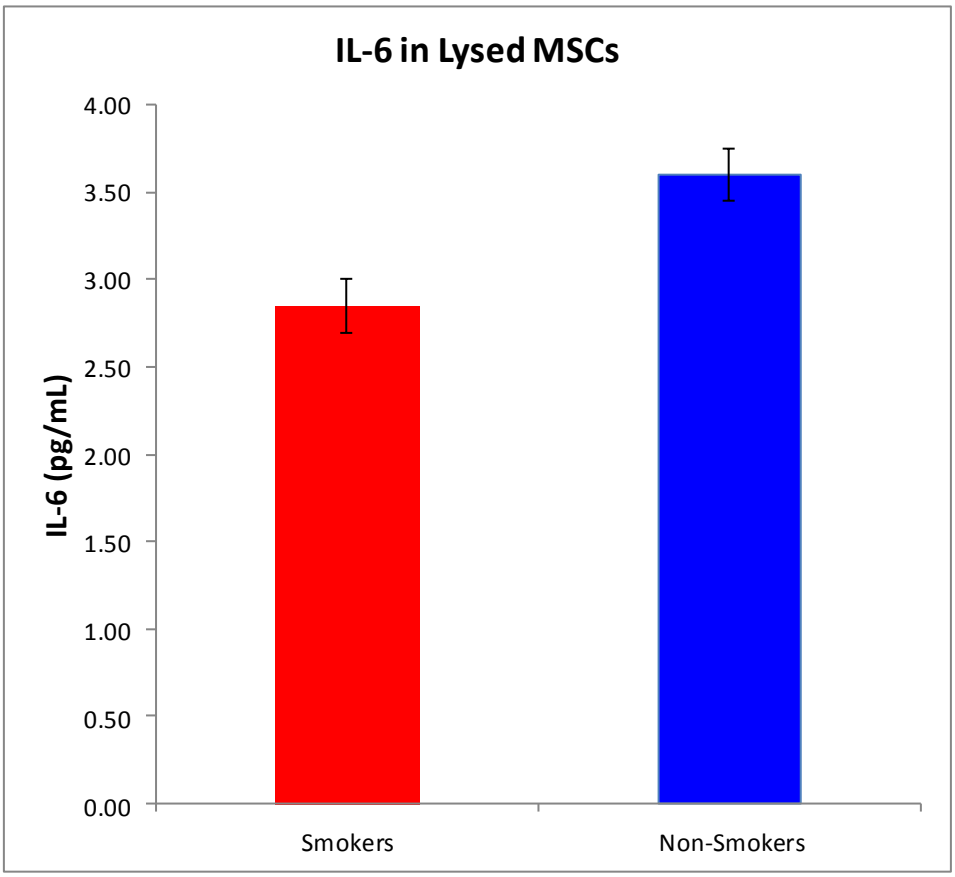


Data not significant ($p>0.05$; Mann-Whitney U test)

Figure 68: ELISA (VEGF-A)

Graph showing levels of VEGF-A in serum extracted from haematoma fractures using ELISA, which were compared between smokers (n=4) and non-smokers (n=5). Values are means with standard error bars (±SEM). This is representative of preliminary data in the study.

3.7.9 Quantitation of IL-6 via ELISA in Lysed MSCs (Smokers vs. Non-Smokers): A Preliminary Study



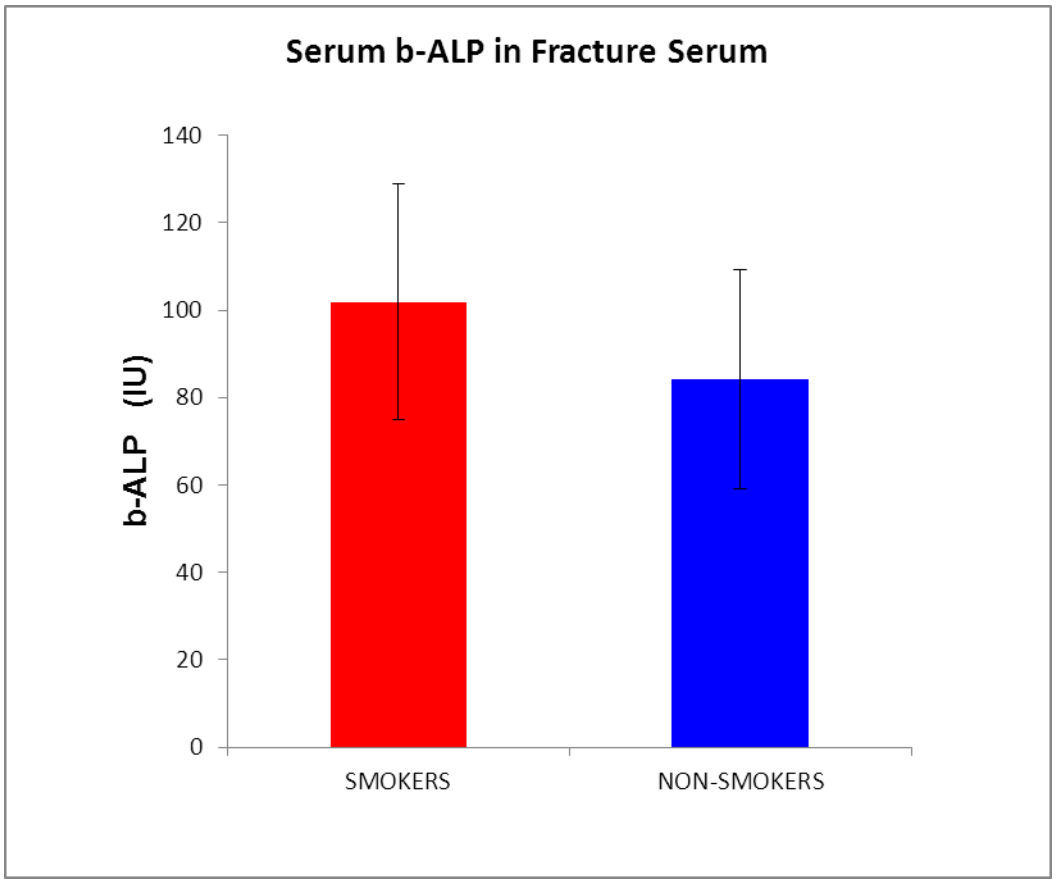
Data not significant ($p>0.05$; Mann-Whitney U test)

Figure 69: ELISA (IL-6)

Graph derived from ELISA for preliminary interleukin-6 (IL-6) quantitation, showing amounts of this cytokine in lysed fracture MSCs, comparing levels between non-smokers (n=5) and smokers (n=5). Values are means with standard error bars (±SEM).

3.8 BIOCHEMICAL STUDIES

3.8.1 Comparison of bone-Alkaline Phosphatase (b-ALP) in Fracture Haematoma Serum in Smokers vs. Non-Smokers

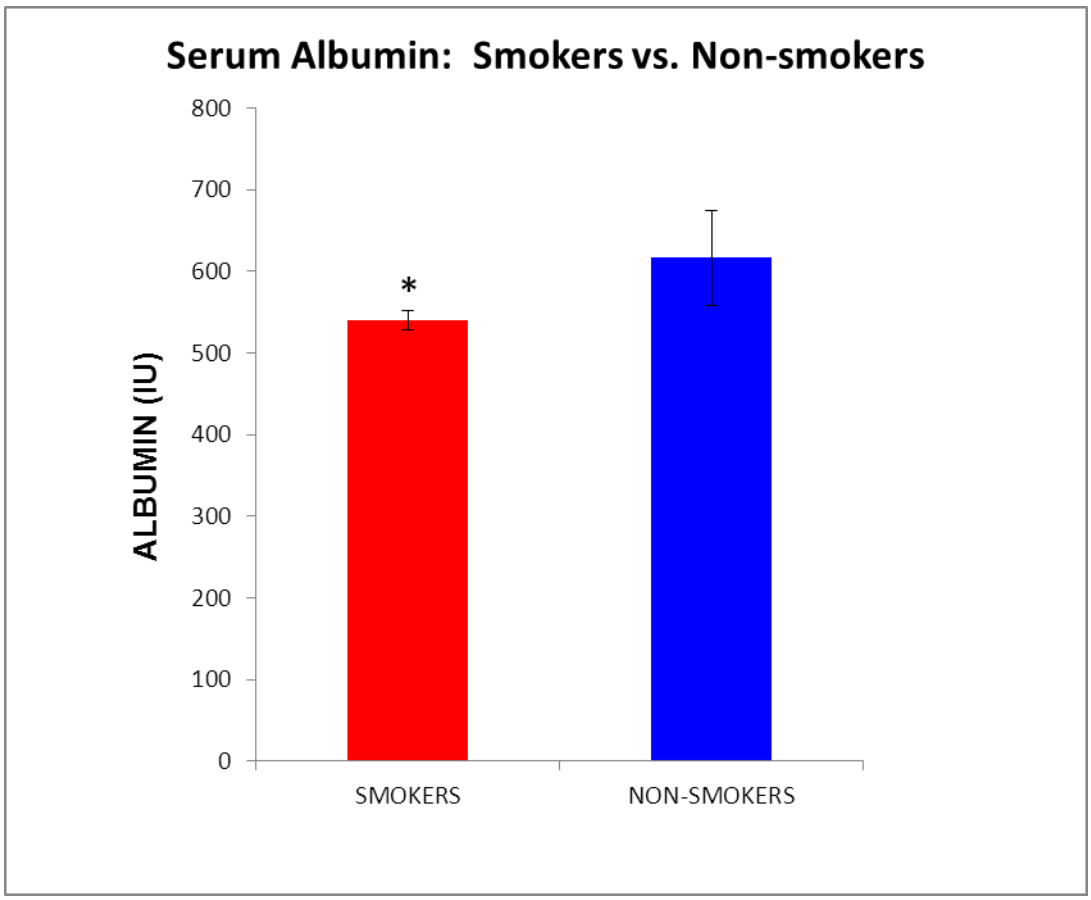


Data not significant ($p>0.05$; Mann-Whitney U test)

Figure 70: b-ALP Levels in Serum

Comparison of bone alkaline phosphatase (b-ALP) levels between smokers (n=10) and non-smokers (n=10) in fracture serum. Normal reference range in peripheral blood: women, 35-104 IU; men, 40-129 IU. Smokers' serum showed higher levels of b-ALP, but a wide variation in data rendered the result not significant. Values are means with standard error bars (±SEM).

3.8.2 Serum Albumin Biochemical Quantitation in Fracture Haematoma Serum (Smokers vs. Non-Smokers)

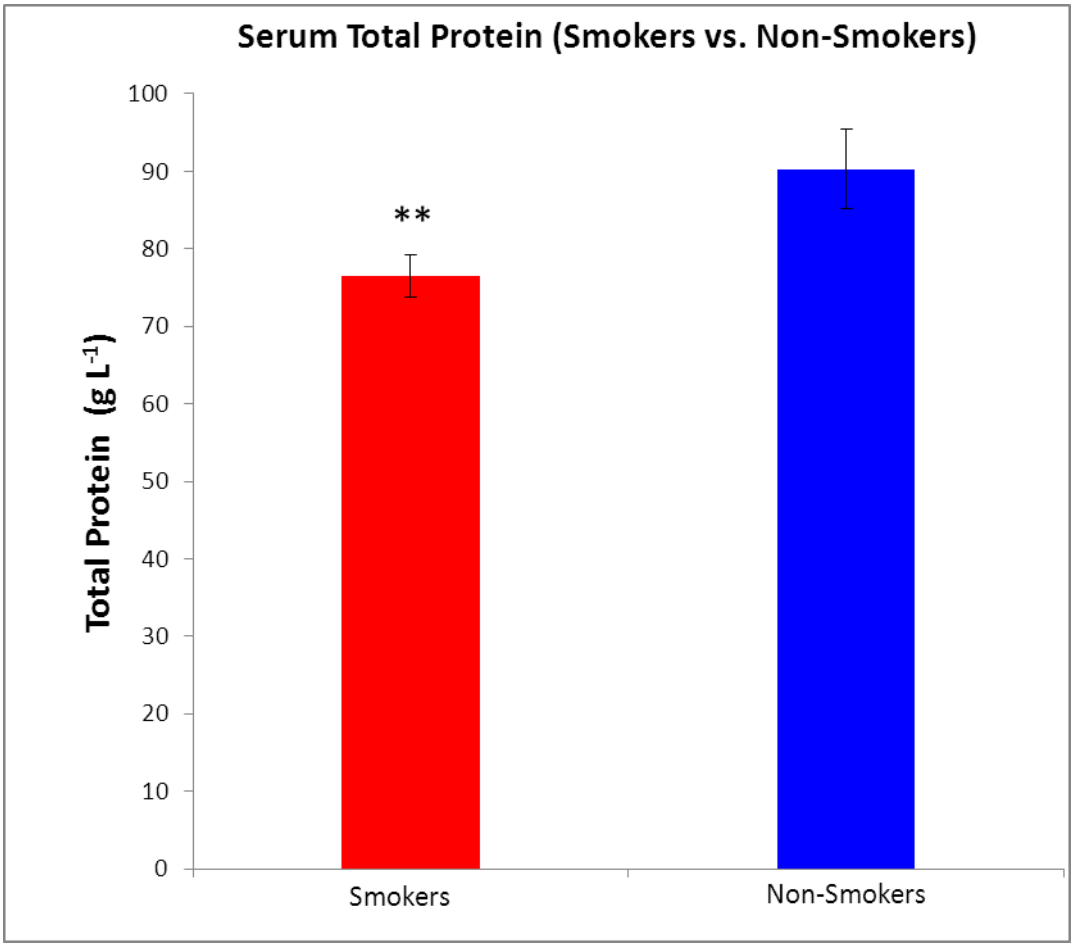


(*p<0.05, Mann-Whitney U test)

Figure 71: Serum Albumin Levels

Comparison of serum albumin levels; smokers vs. non-smokers (smokers (*n*=10) and non-smokers (*n*=10)). Graph shows albumin levels in smokers' fracture haematoma serum is significantly reduced (p=0.016) when compared to non-smokers' levels. Values are means with standard error bars (±SEM).

3.8.3 Comparison of Total Protein in Fracture Haematoma Serum (Smokers vs. Non-Smokers)



(**p<0.01; Mann-Whitney U test)

Figure 72: Total Protein Levels

Comparison of serum total protein (albumin + globulin) between smokers (*n*=10) and non-smokers (*n*=10). Graph shows levels of total protein in smokers' fracture haematoma serum is significantly reduced (actual p=0.007) compared to amounts in non-smokers' serum. Values are means with standard error bars (±SEM).

CHAPTER 4

DISCUSSION

4 DISCUSSION

This study focuses on the clinically-recognised problem of delayed fracture healing in people who smoke tobacco. The convincing findings of orthopaedic investigative groups, such as Adams *et al.* (2001), have demonstrated a significantly-delayed union time in the healing of tibial fractures in human patients who smoke. The literature concerning the *clinical* issues of smoking and fracture healing is relatively more common than the biochemical aspects on human, which are poorly documented. Indeed, inserting 'effects of smoking on fracture healing' into the Pubmed online search engine returns just 68 publications, of which less than 30 are more recent than 5 years old. Those that are available are mainly in the form of reviews, focus upon studies undertaken on animals or are written about the generalised problems associated with fracture healing. The aims of this study were therefore two-fold and were focused on the i) cellular and ii) molecular mechanisms that are relevant in human fracture repair and how smoking can impact on certain biochemical processes. The primary aim was to isolate and study cells, from the fracture microenvironment, particularly those that are directly involved in bone regeneration; the second was to identify and analyse the acute-phase proteins in both haematoma serum and fracture cells that had been extracted from patient samples, with specific regard to making comparisons between smokers and non-smokers. The hypothesis is that cells and acute-phase proteins, the latter produced by the cells and those present in the ECM, extracted and isolated from those patients who were smokers, are inferior in terms of both quality and quantity than those isolated from non-smoking patients.

4.1.1 Clinical Findings from Patient Histories

The patient records were obtained from the hospital registry so as to identify any anomalies that were apparent during the fracture healing process. Of the 48 patients recruited into this study, the majority of fractures healed satisfactorily without any problems. However, there were five patients whose injuries were hampered by certain medical conditions. These idiosyncrasies included nonunion of fracture, obesity, osteopenia, compartment syndrome and Crohn's disease and are discussed here.

4.1.1.1 Smoking-Related Nonunion of Fracture

The radiographs shown in Figures 23 (a), (b) and (c) were acquired from a smoking patient at presentation (a), 6 weeks post fracture (b) and at follow-up, 6 months after external fixation of the tibia and fibula. The patient was a female smoker aged 43 at the time of injury, with no stated underlying comorbidity. Clinical reports state that there was no significant callus seen at 6 months, which is consistent with nonunion. The individual required external fixation using a unilateral frame, which was surgically introduced after it became evident that the fracture was not healing in a satisfactory manner. Interestingly, no cells were expressed from this patient's sample during processing in the laboratory. Indeed, the volume of sample was insufficient for any *in vitro* analysis of any description. The miniscule nature of the haematoma sample likely suggests that there was a general lack of it at the fracture site, which has resulted in nonunion. As it is the callus that is derived from the ECM within the haematoma, then this would correlate with the comment in the patient notes that there was no callus seen even

after 26 weeks post-injury. It would appear therefore that smoking has caused a diminished fracture haematoma in this particular patient. There were no previous comorbidities stated in the medical notes and the individual was otherwise healthy. It is probable that cigarette smoking has been contributory to reduced extravasation at the point of injury due to vasoconstriction, hypoxia and toxicity to the mediators that are involved in haematoma synthesis in early fracture repair. This patient would possibly have benefited from receiving clinical intervention(s), such as BMP-2, to accelerate the rate of healing and stimulation of callus synthesis within the fracture site.

Yu *et al*. (2010) showed that exogenous BMP-2 has the potential to be effective in patients that require clinical intervention to assist in bone regeneration. The group defined the role of BMP-2 during the early stages of fracture repair and how the growth factor can regulate the fate of skeletal progenitor cells in live rats. Fractures were produced in the anaesthetised animals and rhBMP-2 was then injected directly into the fracture site. It was reported that the BMP-2 increased deposition and resorption of cartilage and bone in non-stabilised fractures via endochondral ossification in the rats and also induced cartilage formation in stabilised fractures via intramembranous ossification. It was also shown via immunohistochemical techniques that BMP-2 directed cell differentiation towards chondrogenic lineage in the periosteum and recruited MSCs from the endosteum and periosteum (Yu *et al.*, 2010). The effects of BMP-2 on cultured MSCs was undertaken in this study and the results are discussed later.

4.1.1.2 Obesity

Radiographs shown in Figure 24 (a) and (b) were acquired from a non-smoking, 33 year old female obese patient at presentation (a) and (b) and at follow-up, 6 months after internal operative fixation of the tibia and fibula. The x-ray image (b) depicts a broken screw at the distal portion of the lateral tibia (red circle), around the lateral malleolus anatomically. The medical notes stated that the patient's BMI arose to over 35 in the 6 months following internal fixation, which is classified as Grade II obesity under the Edmonton obesity staging system (Padwal *et al.*, 2011). The internal fixators used in surgical reduction and fixation are composed of either titanium (Yang *et al.*, 2013) or stainless steel (Greene *et al.*, 2008) which has been sheared in this case due to the mechanical stresses that have been placed upon it by the weight of the patient. This patient required revision surgery to reinstate the integrity of the fracture. Ideally, weight-bearing should be undertaken gradually and under rehabilitative physiotherapeutic management, ensuring that the strength of the newly formed bone is sufficient to support the weight of the patient. Obesity can become especially problematic in fracture due to weight gain synonymous with inactivity as a consequence of immobility following leg fracture, a condition that has resulted in healing complications for this individual.

Obesity in fracture has actually been deemed to be beneficial due to the positive effect of mechanical loading afforded by body weight on bone development. This is a controversial research area and many studies support the view that fat accumulation has an injurious impact on bone mass. The likelihood is that obesity affects bone metabolism through a number of mechanisms. Due to the fact that

adipocytes and osteoblasts are both derived from multipotent mesenchymal cells, obesity may increase adipocyte differentiation and fat accumulation whilst decreasing osteoblast differentiation and bone formation. Obesity is also commensurate with chronic inflammation and there is an up-regulation of circulatory and tissue proinflammatory cytokines, giving rise to increased osteoclastic activity and bone resorption via the modification of the receptor activator of NF-κβ (RANK)/RANK ligand/osteoprotegerin biochemical pathway. The increase in proinflammatory cytokine production seen in obesity is caused by excessive secretion of leptin and decreased production of adiponectin, interfering with the mechanisms involved in normal bone formation and resorption. High fat intake also impedes intestinal calcium absorption, thereby decreasing the availability of Ca^{2+} for bone formation (d'Herbomez *et al.*, 2007).

4.1.1.3 Osteopenia and Fracture

The x-ray images seen in Figures 25 (a), (b) and (c) show a fracture from a non-smoking, 84 year old female osteopenic patient at presentation (a) and (b) and at follow-up (c), 6 months after fracture of the tibia and fibula. Whilst there were no complications during the repair process, this condition does pose a high risk factor for fractures, particularly when combined with old age where an increased likelihood of osteoporosis exists, especially in females who are postmenopausal.

Osteopenia is characterised by low bone mineral density (BMD), where the BMD T score is 1-2.5 standard deviations (SDs) below the young adult mean (but is not sufficiently low enough to be classified as osteoporosis, which is 2.5 SDs or more below), as defined by The World Health Organisation (WHO) (Kanis *et al.*,

2008). There is a micro-architectural deterioration of bone tissue, rendering the bone increasingly fragile and at high risk of fracture. Osteoporotic fracture is a major cause of morbidity and mortality and was responsible for 300,000 fractures in the United Kingdom in 2010 costing the National Health Service over 2 billion pounds. The disease is often secondary to underlying factors such as hypogonadism, hyperthyroidism, skeletal metastases, multiple myeloma, old age, alcoholism and cigarette smoking (Tuck and Francis, 2002). There are several biological bone turnover markers that can be used to pathologically diagnose osteoporosis, including bone alkaline phosphatase (b-Alp), osteocalcin (OC), procollagen type I carboxy-terminal propeptide (P_1CP) for bone formation and tartrate-resistant acid phosphatase-5b (TRAP-5b) and cathepsin k that measure osteoclastic enzyme activity in bone resorption (Seibel, 2006; Wheater *et al.*, 2013).

4.1.1.4 Compartment Syndrome in Fracture Healing

Figures 26 (a) and (b) are radiographs taken from a non-smoking, 27 year old male patient, with a history of compartment syndrome and prolonged pain (for 6 months) at presentation (a) and (b) at follow-up, 6 months after internal fixation of the tibia. There were no reported complications in terms of fracture healing during the recovery period of the injury. Compartment syndrome is a severely debilitating, limb- and life-threatening musculoskeletal condition for fracture patients that arises due to an insufficient blood supply to muscle and nervous tissue in 'compartments' such as the arm and leg, leading to increased internal pressure. Extended sessions of surgery (>6h) present a high risk of this sequelae being realised and one which is recognised by theatre staff. Left untreated,

compartment syndrome can lead to severe complications such as rhabdomyolysis (rapid breakdown of muscle tissue) and renal failure (Crawford and Comstock, 2010).

Compartment syndrome can arise in any part of the body where there is little or no room for expansion of muscles; presentations can be acute, sub-acute and chronic. The compartments within limbs contain muscles, arteries, nerves and lymphatic vessels that are tightly packed together and enclosed by an inelastic connective tissue sheath, which limits the volumetric increase of its contents. Intracompartmental pressure (ICP) becomes increased in conditions where there is expansion in the volume of the enclosed tissues, as with fracture or soft tissue damage, or a decrease in the capacity of the compartment itself via, for example, external compression due to plaster of Paris casts and burns (Mabvuure *et al.*, 2012). The most common cause of acute compartment syndrome (ACS) is fracture, accounting for 69% of cases in one particular study (Erdos *et al.*, 2011). Tissue swelling and haematoma formation are known mechanisms responsible for elevating ICP, resulting in ACS (Percival *et al.*, 2011). Any type of fracture can be affected by the syndrome and even open fractures do not relieve sufficient pressure from the skin tear to be discounted from ACS (Elliott and Johnstone, 2003).

There is no formal indication for the management of patients with raised ICP in the United Kingdom, but general practice involves the removal of any dressings overlying the affected compartment, splitting plaster casts but leaving padding intact and elevating the limb to no higher than heart level in order to perfuse the

compartment (Kostler *et al.*, 2004). If clinical approaches fail to relieve symptoms, fasciotomy may be indicated as an emergency procedure but based upon a comprehensive clinical assessment. This is where an incision is made into the 'fascia', or connective tissue separating the muscles from the internal organs, in order to relieve tension and/or pressure (Mabvuure *et al.*, 2012).

4.1.1.5 Crohn's Disease

The x-ray images in Figures 27 (a) and (b) were obtained from a non-smoking, 35 year old male, with a history of Crohn's disease (as seen in the medical notes). Radiographs show fracture at presentation (a) and at follow-up (b), 6 months after internal fixation of the tibia and fibula. The fracture healed satisfactorily, without any difficulties being reported in the medical notes. It is notable that the MSCs derived from the donated sample proliferated rapidly in comparison to cells acquired from other patient specimens. Even though cell doubling time was reduced and 5 day passages typically saw increases in excess of 260% (average 118%), the sample was not the most proliferative, the highest being 380% in non-smokers' cells. However, the yields from cultures were by far the greatest with harvesting after the first passage being over 15,000 cells per μL of sample on the flow cytometer. This compares with an average yield of approximately 360 cells per μL in the non-smoking group. The sample was not therefore considered to be a 'spike' in the normalised data.

Crohn's is a type of inflammatory bowel disease (IBD), resulting in swelling and dysfunction of the intestinal tract. There have been several studies that have reported correlations between Crohn's disease and bone metabolism disorders including osteoporosis and dietary calcium absorption (Zuk *et al.*, 2001; Kumari *et al.*, 2010; Klaus *et al.*, 2011).

One particular study by Vázquez *et al*. (2012) showed that Crohn's disease was a potential risk factor for increased prevalence of vertebral fractures when compared with healthy subjects. The group investigated 107 patients with IBD (Crohn's *n*=53; ulcerative colitis *n*=54) and 51 healthy subjects for morphometric vertebral crush fractures using thoracic and lumbar x-rays of the T4-L4 region. The height of individual vertebrae (from T4-L4) was measured, semi-quantitatively, in each participant and a decrease of more than 20% in the height of any of the bones (>4mm equiv.) when compared to the others was considered to be a morphometric vertebral crush. It was found that there was a higher incidence of morphometric vertebral crush fractures in patients with IBD (44.5%, representing a total of 72 fractures) when compared to the control group (13.7%, representing 10 fractures) ($p<0.0001$) (Vazquez *et al.*, 2012).

4.1.2 Sample Collection for *In Vitro* Studies

The sample collection incorporated the clinical aspiration of a small amount of the fracture haematoma (typically 2.0-4.0 mL) from tibial fractures of anaesthetised patients during the surgical fixation of the damaged bone; tissue which is said to be normally washed away by the surgeon during the reduction and nailing

procedure. The tissue was processed for analysis in the laboratory and the two basic intentions were to extract mesenchymal stem cells and to acquire serum from the haematoma; the interstitial fluid that surrounds these cells at the fracture site that forms much of the extra cellular matrix. The methodology regarding stem cell analyses from fracture haematomas was first published by Oe *et al.* (2007), who were successful in characterising what were referred to as *progenitor cells* from human fracture haematomas. Their work was pioneering and there are very few comparable publications currently available that emulate their work in the field. However, the presence of non-haematopoietic cells in bone marrow is not a new concept, as it was first reported by the German physicist Conheim, over 130 years ago, that bone marrow is a rich source of fibroblasts that deposit collagen fibres during normal wound repair (Prockop, 1997). Therefore, for the cellular aspects, the objective was to obtain cells from the donated tissue that are relevant in bone repair; mesenchymal stem cells, so-called due to their ability to differentiate into mesenchymal-type cells (Chamberlain *et al.*, 2007).

4.2 Cellular Analysis of Fracture Healing

Cell culture is necessary in order to isolate cells of interest that have properties which allow them to be readily studied *in vitro*; their ability to divide rapidly ensures a constant availability in the liquid nitrogen. As previously stated, Oe *et al.* (2007) successfully isolated mesenchymal stem cells from human fracture haematomas and demonstrated their renewable capabilities *in vitro*. The identity of these cells was confirmed by undertaking immunocytochemical assays that involved the binding of specific antibodies to MSC surface antigens and the

techniques that were used by Oe and co-workers formed the basis of the characterisation work in this current study. After extraction and population doubling, the group were able to differentiate haematoma cells into adipose, muscular, bone and cartilage tissue *in vitro* by using specialised differentiating media. The cells were reported to have a good viability for subculture, *in vitro* analysis and differentiation. Indeed, Oe and colleagues stated that cells of this nature could be cultured through at least 10 passages with minimal decline in proliferative capacity or volume. It was therefore deemed in this study that comparable cells extracted from donated human fracture haematomas were suited to laboratory-based investigation.

4.2.1 Cell Explantation

In the primary cultures, excised portions of the fracture haematoma were explanted and incubated and left undisturbed, for 7 days, a process based on methods described by Niikura (2005) (Niikura *et al.*, 2005). Ordinary light microscopy was used to study the unstained primary cultures, which were observed as mixed populations of cells and other material within the tissue culture flasks. Not all samples, however, bestowed the desired cell type and, unfortunately, unsuccessful attempts outnumbered successful extractions over the course of the study (see Table 9, Ch. 2). Methodologies involving the direct explantation and Ficoll Paque centrifugation proved to be the most effective in extracting progenitor cells and as such were both adopted as the standard procedures in which cells were procured from donated tissue. Sample quality often differed across specimens, rendering the consistency to be unpredictable.

Smokers' cells were particularly difficult to extract in the laboratory, mainly due to both the low frequency of donations and, ironically, their lack of quantity *and* quality when compared to the non-smokers' specimens, perhaps a valid observation in itself. This is reflected by the low sample sizes in some of the cellular experiments, which did eventually extend to n=7 for smokers and n=11 for non-smokers. When samples did express cells, confluent, localised pockets of adherent, fibroblast-like structures were observed, which appeared to be migrating from some of the explanted haematoma tissue in the culture flasks and which were adherent to the base of the polystyrene tissue culture flasks (see Figures 28-34). The commercial mesenchymal stem cell line was also cultured in the same way as the cells derived from the fracture haematoma and displayed a very similar morphology under light microscopy (Figure 34).

4.2.2 Adherent Properties of Fracture Cells in Culture

Cell adherence is bridged by amphoteric proteins and the prerequisite for the attachment onto an *in vitro* substratum is due to the adsorption of proteins and other factors to the culture surface. The substratum on which cells grow (for example, polypropylene plastic, polystyrene or collagen) possesses either a net negative or positive charge. The charge density on these surfaces is responsible for attachment and spreading of cells rather than their polarity. It is also known that cell adhesion is regulated by specific cell surface receptors. The cells become attached to the substratum before producing their own glycoproteins and matrix proteins prior to spreading-out. It is this matrix that adheres to the charged surface and the cells then become bound to the matrix via specific receptors.

Surfaces, or substrata, that have been conditioned by previous cell growth are often said to provide a better surface for attachment, but this was not adopted in this study (Mukhopadhyay *et al.*, 1993).

Cell adhesion also depends on a functional contractile system, involving multiple contacts with the surface. Lamellipodia structures, which are filled with bundled actin filaments, fit into a lattice structure formed by the glycoproteins on the substratum. This is followed by cytoplasmic webbing and flattening of the cell mass (Hirtenstein *et al.*, 1980). Once the cells became anchored to the substratum, they grew and consumed the nutrients that were available to them in their culture medium, including the infused CSE toxins, applicable to a number of experiments in this study. The liquid culture media (IMDM) has an ambient pH of 7.4, contains carbon and nitrogen, an energy source (L-glutamine), growth factors within foetal calf serum (FCS) and dissolved oxygen and other gases, all of which are essential for cell sustainability and development. An anti-fungal agent and antibiotic were also present to maintain the health of cells, although overuse was avoided since continuous application of certain antimyotics could have a detrimental impact due to toxicity. The lifespan of cultures can be compromised by media degradation, nutrient limitation, the accumulation of toxic metabolites and also infection, which can be fungal, bacterial and/or viral. Other important considerations are environmental factors such as humidity, CO_2 levels and correct temperature (Schmid *et al.*, 1992). After successful cellular proliferation had been achieved, it was necessary to harvest and give a formal and scientific identity to the cells.

4.2.3 Characterisation of the Haematoma Cells

After a period of approximately 26 days from initial plating, the adherent cells were dislodged from the substratum using 0.25% trypsin containing 0.02% EDTA (Sigma-Aldrich, Gillingham). The adherent, fibroblast-like cells that were isolated from fracture haematomas *in vitro* were characterised, mainly as MSCs, via immunofluorescence and immunophenotyping (see Figures 35-48). The cells expressed antigens specific to CD29, CD44, CD105 and CD166 in the immunocytochemical assays, which formed both qualitative and quantitative analyses. The haematopoietic stem cell marker, CD34, used as a negative control, was also detectable in varying degrees throughout passages and, as with the immunofluorescence assays, un-probed cells showed minimal autofluorescent characteristics. The type of cell that is associated with the CD34 cell surface antigen was not of interest because it does not have a major role in the *early stages* of fracture healing (although it is said to be involved in bone osteoclastic remodelling in the latter stages of fracture repair (Pagliarulo *et al.*, 2008)) and was therefore considered to be an undesirable contaminant in the MSC cultures. After four passages, the presence of the haematopoietic surface antigen marker, CD34, was reduced from 69.39% in the first passage to 5.98% in the fifth, suggesting that the non-adherent, haematopoietic, CD34+ cells were voided during routine changes of media during *in vitro* development. Additional immunocytochemical control studies were performed on cyto-spun cells (where no antibodies were used as probes) to demonstrate that cells were not merely auto-fluorescing in the original assays that did include the MSC biological markers (see Figures 40, 41 and 48).

Human MSCs, sometimes referred to as marrow stromal cells, are capable of becoming differentiated into osteoblasts, chondrocytes, adipocytes, myoblasts and possibly neuronal-like cells and are therefore essential in fracture healing. During *in vitro* culture, the haematoma cells self-renewed and divided quickly, having an estimated population doubling time (PDT) of around five days for non-smoking (or non-CSE treated) cells. This PDT correlates with data reported by Azouna *et al.* (2012), who also reported *in vitro* bone marrow-derived MSC mean doubling times of five days (Azouna *et al.*, 2012). The smokers' (and CSE treated) cells proliferated more slowly (discussed later). The rate of proliferation depends on donor, initial plating density (inoculum) and numerous environmental factors. Cell yield can also be lowered via cryogenic storage conditions and mechanical stresses being placed upon them such as centrifuging. Chemically-toxic reagents used in experiments, e.g. trypsin and EDTA, can also have a negative impact on cell number, so there may be some discrepancies with MSC doubling times reported in the literature. More frequent counting, taken place daily, could give a more accurate indication of cell PDTs. The FITC- and PE-conjugated antibody markers described here are not truly specific to MSCs, although it is agreed in the literature that these cells types do not express the antigens specific to the haematopoietic CD markers CD11, CD14, CD34 or CD45. They do, however, express CD29, CD44, CD71, CD73, CD90, CD105, CD166, Stro-1 and intracellular adhesion molecule-1 (ICAM-1) (for general summary, see Table 4, Ch.1) (Chamberlain *et al.*, 2007).

It has also been proposed by the Mesenchymal and Tissue Stem Cell Committee, a subdivision of the International Society for Cellular Therapy (ISCT), that MSCs used for laboratory-based scientific investigation should possess the following three criteria:

- Adherence to plastic
- Specific surface antigen (Ag) expression
- Multipotent differentiation

The criteria for adherence to plastic must be seen in standard culture conditions using tissue culture flasks. Regarding surface antigen expression, ≥95% of the MSC population must express the aforementioned CD surface antigens and be ≤2% positive for a number of haematopoietic CD markers, including CD34. The final stipulation is that the MSCs must be able to differentiate to osteoblasts, adipocytes and chondroblasts (Dominici *et al.*, 2006). In the current study, cell adherence was well established, so has met the first criteria as listed above. The cells expressed well over 95% of the cell surface antigens named, with the exception of CD105 (94.44%), but the lowest recorded CD34 expression was 5.98% (see Table 10 for details). The CD105 antigen seemed to be the most problematic surface antigen to achieve purity, but was nevertheless very close to the 95% minimum threshold as stipulated by the ISCT, in spite of the fact that a 'cell sorter' had not been available at any time in this study. The CD34 expression appeared to compliment the CD105 antigen and it can be noted from Table 10 that early passages showed low levels of CD105 and high levels of CD34, totalling approximately 100% when added together. In later passages, there was a reversal of this phenomenon as non adherent cells were washed out of

tissue culture flasks and the adherent cells proliferated, which forms an interesting observation regarding the cross section of cell populations; simply put that adherent cells are proliferating, whilst non-adherent cells are being expelled from flasks during media changes. A lingering 'background' CD34 expression in later cultures was observed and was likely due to a small proportion of haematopoietic cells having adherent properties, as noted in the literature (Pagliarulo *et al.*, 2008). A diminishing CD105 expression in the later passages was also noted in Table 10 and could be owed to either a temporal-induced reduction in the expression of this particular antigen in subsequent cultures, or just simply that the quality of the FITC antibody reagent was deteriorating over time and therefore more difficult to detect by the flow cytometer. The third ISCT stipulation (multipotent differentiation potential) was not performed as part of this study, mainly due to costs, but also because it has been reported in the literature and would be an experiment to be undertaken in future studies. Oe *et al.* (2007) showed that cells extracted from fracture haematomas were capable of *in vitro* differentiation into osteoblasts, adipocytes and chondrocytes, thus meeting a capacity for tri-lineage mesenchymal differentiation. It is therefore feasible that the cells isolated from patient fracture haematomas in the current study are indeed, beyond reasonable doubt, stem cells of a mesenchymal lineage that are involved in the early stages of fracture healing.

4.2.4 Cell Counting

The cells were cultured through several passages and counts were performed at the end of each, usually as confluent colonies. *In vitro* proliferation rates were

compared between smokers and non-smokers after five days (non-parametric significance testing using the Mann-Whitney U Test), to assess differences that may reflect the cellular damage caused by smoking. It was found that the cells extracted from smokers, as well as those treated with cigarette smoke extract (CSE), proliferated more slowly than the non-smoker and non-treated cells. However, unlike the direct smokers vs. non-smokers comparisons using independent samples, the CSE assays (parametric significance testing via ANOVA) showed a significant difference ($p<0.05$), which was likely due to reduced variance in the results and possibly equal group sizes resulting in normal-distribution of data. However, if the patient comparisons had been reported as $n=7$ for both groups, then a significant difference would have been achieved ($p<0.05$, compared to the reported $p=0.17$), as the variance was improved.

A similar study by Wong and Martins-Green (2004) focused on how infused cigarette smoke affected the survival and migration of chicken embryo fibroblast cells (CEFs) *in vitro*. These galline cells were exposed to measured doses of cigarette smoke components that were comparable to the levels of circulatory serum nicotine found in regular smokers (0.04 mg mL^{-1}-0.07 mg mL^{-1}) (Russell *et al.*, 1980). It was found that the smoke, whilst not killing cells, did inflict a detrimental effect on health by stimulating the production and activation of intracellular stress-response proteins. When allowed to recover, the treated cells divided significantly slower ($p<0.05$) than those fibroblasts not previously exposed to cigarette smoke, which was also shown in our study on the human MSCs that were infused with CSE (see Figure 56). Wong and Martins-Green (2004) also reported damaging alterations in the cytoskeleton and cell adhesion

molecules, giving rise to abnormal inhibition of cell migration, which is essential for proper wound healing and closure (Wong and Martins-Green, 2004). This was perhaps replicated on the fracture MSCs, as the decline in proliferation rates were much lower in smokers' cells compared to non-smokers' cells after 3 passages, thus demonstrating the serious cell damage that is caused by tobacco smoking.

4.2.5 The Infusion of Cigarette Smoke Extract (CSE) into MSC Cultures

The cigarette smoke extract (CSE) was introduced into the study as a means to incorporate a synthetic model of cigarette smoking that was similar to the one used by Wong and Martins-Green (2004) and was itself based on a protocol published by Bernard *et al.* (2004). A lack of available haematoma samples, in addition to sporadic frequency and patient anamnesis, may also have necessitated the introduction of this model in order to project the possible effects of smoking ahead of collecting the required amount of samples needed to produce robust conclusions. It was also important to incorporate the model to isolate smoking as a singular cause of cellular damage, since it is known that other factors can contribute to fracture malunion, including obesity, malnutrition, iatrogenic pathologies and old age (Massari *et al.*, 2013). Proliferation rates showed that the CSE was having a significant toxic effect by slowing down growth and development, or even perhaps causing the death of cells in culture, when smoke toxin levels were equivalent to a person smoking 20 cigarettes per day (equivalent 2.5% CSE in the culture media). In addition to proliferation rates, nitric oxide levels were found to be lower in the treated cells and necrosis was higher with CSE infusion. The damaging impact of the CSE was significant in all the assays

where it was included and showed that so called 'average' levels, equating to a person smoking twenty cigarettes per day, were enough to harm cells in a way that would definitely compromise the development of cells that are involved in the fracture healing process.

4.2.6 Cellular Nitric Oxide (NO) Quantitation

Nitric oxide is a free radical gas which is known to be a secondary messenger metabolite in various biochemical pathways and has been implicated in a number of orthopaedic pathologies, including inflammation, osteoporosis, arthritis and aseptic loosening (Kaur and Halliwell, 1994; Stefanovic-Racic *et al.*, 1994; Sakurai *et al.*, 1995; Wimalawansa *et al.*, 1996; Hukkanen *et al.*, 1997). NO is synthesised by the conversion of the amino acids L-arginine to L-citrulline; catalysation is achieved via a group of isoenzymes consisting of endothelial nitric oxide synthase (eNOS type III), neuronal NOS (nNOS type I) and inducible NOS (iNOS type II). The former two are thought to occur naturally; they are calcium-dependent and are essential in generating basal levels of NO in homeostasis. Synthesis of the latter is accomplished by proinflammatory cytokines and is reported to be calcium-independent; indeed iNOS is frequently present in pathologies when induction results in the production of large amounts of NO. The widely-held view advocates that NO production by endothelial cells is affected by cigarette smoking, which is believed to interfere with endothelium-dependent vasodilatation in a range of vessels (Barua *et al.*, 2001).

Notwithstanding this, nitric oxide has a major role in fracture healing and is widely reported to have a major effect on osteoblasts, osteoclasts and osteocytes, with eNOS and iNOS having significant cell signalling roles in osteoblastic proliferation and function (Ralston *et al.*, 1994; Hukkanen *et al.*, 1995; Riancho *et al.*, 1995). Conversely, iNOS has been shown to have an inhibitory effect on osteoblastic cultures and studies have shown that cytokine-induced iNOS activity, associated with high NO production, causes a *reduction* in osteoblastic proliferation and differentiation, in addition to triggering apoptotic activity and is relevant in late fracture healing (Hukkanen *et al.*, 1995; Corbett *et al.*, 1999). Osteoclastic activity is also known to be suppressed by iNOS (Brandi *et al.*, 1995). These characteristics give further weighting to NO's regulatory role in fracture healing in both the early and late stages of fracture repair (Paskalev and Goranov, 2012).

Nitric oxide is, therefore, a mediator of osteocyte function and epiphyseal bone remodelling. NOS inhibitors, such as N^G-nitro-L-arginine methyl ester (L-NAME), are known to reduce bone turnover and repress mechanically-induced bone formation, implicating NO as an early mediator of increased bone formation. Higher levels of the metabolite present in osteoblasts would suggest more effective bone repair (Fox *et al.*, 1996).

A vascular response is required in bone repair and there is a significant increase in blood flow at the site of injury following fracture, combined with hyperactive vascular formation prior to the development of the fracture callus. Nitric oxide has been described as an endothelial-derived relaxing factor (EDRF) and is a

mediator of endothelial-dependent vasodilatation. It has also been stated that surrounding bone, smooth muscle, vascular endothelial cells, chondrocytes and certain leukocytes are all sources of NO (Corbett *et al.*, 1999).

Corbett *et al.* (1996) investigated whether NOS isoforms were expressed during the healing of fractures. Unilateral tibial fractures were made in male Wistar rats (n=100) and comparisons were made between fracture and normal bone. Immunohistochemistry showed an increased expression of eNOS, which was strongest in the cortical blood vessels and osteocytes in the early phase of fracture repair. Western blot and image analysis confirmed the increase. They observed significantly-raised calcium-dependent NOS activity 1 day after fracture ($p<0.05$). Inducible NOS was localised in osteoblasts and chondroblasts in the second week of fracture healing. Western blotting showed a significant, 3.64-fold reduction in iNOS during the early phase of healing compared to normal bone. The group also showed a significantly-reduced calcium-independent NOS activity, but neuronal NOS was not detected. It was stated that increased eNOS in bone blood vessels is likely to mediate the increased blood flow that is associated with fracture repair. Their results showed that eNOS was increased and iNOS reduced in early healing of fractures and it was concluded that this is an important factor in successful fracture repair.

Nitric oxide was shown to be significantly reduced in the cells of CSE-treated cultures in the current study ($p<0.05$). It is not known which of the NOS isoforms this represents; this would need to be determined using specific ELISA quantitation, but nevertheless shows that smokers are likely to have lower

amounts of nitric oxide at the fracture site compared to non-smokers. It is therefore apparent that non-smokers would benefit from higher levels of eNOS than smokers, because of increased vasodilatation and calcium availability, in addition to increased osteoblastic and decreased osteoclastic activity, as suggested by Corbett *et al.* (1999). It is also indicative of a reduced level of iNOS in smokers, demonstrating retarded cytokine mobilisation, coupled with abnormal osteoblastic/endothelial hyperactivity, strengthening Wong and Martins-Green's (2004) theory of tobacco-induced, abnormal over-healing and fibrosis in fracture repair, likely due to reduced apoptosis of mature bone cells, giving rise to chronic cellular aging. Indeed, such a lack of eNOS intervention could result in hindered apoptosis and increased osteoclastic activity with reduced angiogenesis, therefore having a detrimental impact on the bone repair process due to a deficiency of NO-led mediation in smokers.

Nitric oxide is indeed integral in fracture repair, as it impinges on the metabolism of osteoblasts, osteocytes and osteoclasts, which is vital for the successful derivation of the fracture callus seen in bone repair. Lower levels of NO observed in CSE-treated cells suggest that smokers are therefore deprived of proper vascularisation and vasodilatation, have decreased cytokine and calcium involvement, a limited capacity for adequate cellular organisation in early fracture healing, as well as impaired bone remodelling in the longer term.

4.2.7 Cellular Necrosis in MSC Cultures

Apoptosis is a process that is comprised of a scheduled and actively-coordinated series of metabolic events within cells. Necrosis, on the other hand, is a passive response to a cytotoxic or injurious milieu that leads to uncontrolled cell death. In apoptotic circumstances, the cell membrane remains intact until late into the process, which is unlike necrosis, when permabilisation of the phospholipid bilayer occurs in the very early stages. Propidium iodide is a chemical probe that was used during flow cytometry to quantitatively measure the amount of double-stranded nucleic acid present within a cell. Since it cannot pass through an intact cell membrane, which acts as a barrier, propidium iodide can be used to test the viability of cells, i.e., it does not fluoresce if added to a suspension of intact cells. It is, for these reasons, able to cross the cell membrane of necrotic cells due to membrane 'flipping', interact with double-stranded DNA, whereby red fluorescence is detected by the flow cytometer. Propidium iodide is a stain that was used to mark necrotic cells in this study (see Figure 58), as cells with large enough holes in their membranes to allow the penetration of propidium iodide are dead and cell viability can be measured in the presence of various toxic conditions (Givan, 2004), as was the case when infusing MSC cultures with CSE.

In the cell necrosis assays using propidium iodide, cell death caused by smoking was analysed in CSE-treated cells, with untreated cultures being used as a control. It was found that, after 4 passages, the cigarette smoke caused significantly more necrotic cell death than was occurring in the untreated cultures ($p<0.05$). This showed that the CSE was having an injurious effect on cellular viability and was probably responsible, in part, for causing a reduction in cellular proliferation as

seen earlier-on in this study. However, what was notable regarding the results was that there appeared to be an initial increase in necrotic activity in both groups, between the first and second passage. This early increase can be seen as a 'carbon copy' in the graph, before a divergence into the third passage, showing a mutual decline in the amount of necrosis taking place before rising again with a greater degree of separation into the fourth passage. The initial increase is likely due to there being some residual dead cells from the freeze/thaw process after samples were taken out of the liquid nitrogen for analysis. It is also likely that dead cells, which lose their adherence properties, were being discarded during routine media changes and that cell proliferation resumed thereafter due to there being a healthy and well-established population of cells which expanded throughout later passages. As cells increase in number and size, they take up more surface area in the flasks, become older and are perhaps more prone to mechanical stresses *in vitro*, all of which would contribute to higher levels of necrosis generally. There could also be some late-stage apoptotic activity. That accepted, the CSE simply served to further aggravate and amplify the necrotic processes seen in the CSE-treated cultures. The toxic attributes of cigarette smoke *in vivo*, therefore, may influence premature cell death in patients who are smokers. This leads on from the previous discussion concerning nitric oxide, where it was stated that smoke placed inhibitory forces on apoptosis. It has now been shown that necrosis is more prevalent and plausibly impinges on the synthesis and development of premature osteoblasts in early fracture repair, whilst natural and programmed cell death is reduced in more matured bone cells. This thereby affords fortification to the argument that smoking unbalances the natural turnover of cells that are involved in remodelling by causing increased necrosis and reduced apoptosis in

fracture healing in smokers. To complement this experiment, future work may investigate the effect of smoking on apoptotic activity.

4.2.8 Cell Recovery Assay

The cellular assays have thus far focused upon how cigarette smoking can impact upon cellular development. These findings therefore raises the question; does the cessation of tobacco smoking improve cellular function and proliferation in fracture healing? The cellular recovery assays does suggest this to be the case, as cells *in vitro* were shown to recover after CSE withdrawal from cultures. Morphological studies were undertaken initially when using the smoke extract and cells were observed to be fragmenting after its infusion. When the CSE was omitted from culture media, cells resumed the classic fibroblast-like, spindle-shaped structure. This suggests that once CSE is withdrawn, it is likely that cells repair themselves in the media and recommence proliferation with reduced cellular death in populations. These observations led to a more formal assay that was designed to quantitate proliferation studies, which was conducted over three weeks. It was then clear that the withdrawal of CSE after a five-day infusion again improved cellular development and proliferation levels were markedly enhanced after 21 days of CSE omission in the culture media. This data, however, was not significant due to considerably-overlapping standard error bars on the graph, relating to a substantial level of variance across small groups, which impacted on the SEM within the data. The simple fact that cells are derived from individual patients and not from a commercially-available cell line, is probably due to the occurrence of this phenomenon regarding the wide variation. In order

to minimise the variance and hence gain a greater degree of significance, more samples would need to be incorporated into the study. In similar work published in the literature, scientists have used much larger sample sizes to reduce variance and achieve a more robust level of significance in studies, e.g. Corbett *et al*. (1999) conducted research using 100 rat specimens, Adams *et al*. (2001) with a cohort of 277 human subjects, whilst Frost-Peneda *et al.* (2010) performed research on 3,585 adult smokers and 1,077 non-smokers (Corbett *et al.*, 1999; Adams *et al.*, 2001; Frost-Pineda *et al.*, 2010). As quoted by Cumming *et al*. (2007) in *The Journal of Cell Biology*, 'It is highly desirable to use a larger *n*, so as to achieve narrower inferential error bars and more precise estimates of true population values' (Cumming *et al.*, 2007, pg. 7). It would therefore be imperative to recruit more patients if future studies are to be considered and ideally, from additional clinical sources to increase donor frequency.

4.2.9 *In Vitro* Effect of Bone Morphogenetic Protein-2 (BMP-2) on Cell Viability via the MTT Assay

The application of soluble growth factors, such as BMP-2, has been the subject of debate largely due to its suitability in the field of bone tissue engineering (Wozney, 2002; Turhani *et al.*, 2007). As members of the TGF-β superfamily, BMPs are principally differentiation factors and regulators of the bone induction cascade in skeletal tissue regeneration (Wikesjo *et al.*, 2004). The growth factor BMP-2, which is up-regulated in the early stages of fracture healing (see Table 6), can be administered exogenously (as recombinant BMP-2 (rhBMP-2)), via a delivery vehicle, to various sites in order to accelerate bone regeneration. It was

shown in one *in vivo* study to enhance bone formation in bone gap healing (Yudell and Block, 2000) and in another to improve osseointegration of implants when covalently bound to biomaterial surfaces (Schmitt *et al.*, 1999).

Turhani *et al.* (2007) investigated the supplementation effect of increasing concentrations of rhBMP-2 *in vitro* on the osteosarcoma cell line with a proven SaoS-2, which were derived from osteogenic sarcoma and have osteoblastic phenotype characteristics. In the 2007 study by Turhani *et al*, cell cultures were subjected to various dose concentrations of exogenous rhBMP-2 (10 ng mL^{-1}, 100 ng mL^{-1} and 500 ng mL^{-1}) and analysed after 24h, 48h and 72h for viability via conversion of the tetrazolium salt, XTT. As only viable cells convert XTT to the coloured product and conversion is directly proportional to the number of viable cells, absorbance from the converted XTT solution was read on a plate reader at 450 nm. The group found that there was a dose-dependent increase of cellular viability from infusions of rhBMP-2 that was increasing in significance over time, so that after 72h, 10 ng mL^{-1}; $p<0.05$, 100 ng mL^{-1}; $p<0.01$ and 500 ng mL^{-1}; $p<0.001$. However it was concluded that 'further investigations were needed to elucidate the intricate regulatory mechanisms influencing cell responses to exogenous growth factors' (Turhani *et al.*, 2007, pg.492).

The MTT cell viability assay that was undertaken in our study was aimed at replicating the work of Turhani *et al*. (2007), but with the addition of CSE to cultures so as to synthesise an *in vitro* smoking environment. The work carried out on the SaOS-2 was emulated in the current study as it was the most closely-related cellular work available in the literature. The proposal was that exogenous

infusions of BMP-2 could ameliorate fracture repair in smokers by accelerating the growth and proliferation of osteoblasts and be therefore deemed as a possible osteoinductive therapy to smokers who are displaying symptoms of malunion. The same dose concentrations used by Turhani and co-workers were replicated and cell viability was analysed at the same time intervals. However, enhancements of BMP-2 cell viability showed no notable response until the 72h point. This suggests that a cigarette smoke-induced toxic environment was likely to have impeded the early activity of the growth factor, implying that it perhaps has a limited effect on smokers' cellular development in the preliminary stages. Despite this, all concentrations at the 72h point did appear to have a beneficial effect when compared to the control group (that did not receive any BMP-2, but was infused with CSE). Regardless of the apparent improvements attributable to BMP-2, the smallest dose of 10 ng mL^{-1} was the only dose concentration that appeared to have a statistically-significant effect ($p<0.05$) on improving cell viability when significance testing was performed using the Student's *t*-Test. This was, however, not mirrored by the control study, where the impact of exogenous BMP-2 had a much more significant response in the higher dose group of 100 ng mL^{-1} ($p<0.00001$) on untreated MSCs, with the lowest dose of 10 ng mL^{-1} being insignificant.

However, it was later found that using the Student's *t*-Test in the 72 hour CSE MTT study returned a type I error for this data. When using ANOVA statistical testing it was found to be not significant ($p>0.05$). Committing a type I error has therefore produced a false positive in the data, and is probably due to a small sample size, resulting in low power of the test. A prospective power analysis

would be useful in determining the optimal sample size required in the assay to boosting the level of statistical power in the test. The following equation can be applied:

$$\text{Necessary Sample Size} = (\text{Z-score})^2 - \text{SD} \times (1\text{-SD}) / (\text{margin of error})^2$$

(Smith, 2013)

Tabulated Z score for 95% confidence interval = 1.96

Margin of error (confidence interval) = +/- 5% (0.05)

Where SD is the standard deviation. For the 72 hour CSE MTT pilot study (SD = 0.188 for 10 ng mL^{-1}), this is as follows:

$$((1.96)^2 \times 0.188(0.188)) / (0.05)^2$$

$$(3.8416 \times .0353) / 0.0025$$

$$0.136 / 0 .0025$$

$$54.4 = 55 \text{ respondents are needed}$$

The equation has shown that 55 respondents would need to be recruited into the study to optimise the statistical power of this particular assay for 10 ng mL^{-1} BMP-2 treated cells, assuming a confidence interval of +/- 5%. This is equal to an additional 45 non-smoking patients' samples, assuming that there would be a 100% success rate in the isolation of cells from the fracture haematoma. However, it would be advisable to repeat the pilot study to ensure reproducibility prior to a larger scale study.

The original work by Turhani *et al*. (2007) suggested that the higher the dose concentration, the greater the benefit to cell viability, but this was not the case in our particular assay where CSE was infused as an intervention. The reason for this is unclear, but it may be that a dose concentration of 10 ng mL^{-1} was sufficient to saturate the cell inoculum of 3.0 x 10^3 per well and that using increased amounts led to an antagonistic, rather than the intended agonistic effect and actually administering too much BMP-2 could have a cytotoxic effect *in vitro*. It would be therefore beneficial to repeat this assay using a larger sample size, likely narrowing variance and increasing the robustness of data. In addition, time increments could be extended to allow the growth factor to reach its optimal effect; using a larger inoculum should also be considered. Other relevant exogenous growth factors may perhaps be incorporated into the study (as listed in Table 7, Ch. 1), in order to determine the efficacy of those which have the greatest potential in addressing fracture malunion in patients.

4.3 THE MOLECULAR ASPECTS OF FRACTURE HEALING

The haematoma is known to contain and array of acute-phase proteins and MSCs which are the *molecular* mediators of the inflammatory response and subsequent healing process (Oe *et al.*, 2007). In order to study the interstitial growth factors and cytokines known to be present at the fracture site (i.e., the molecular aspects), the haematoma serum was extracted from the donated tissue for analysis, thus representing the extracellular matrix (ECM) within the fracture milieu. The ECM has an intimate involvement with all aspects of bone remodelling, especially fibrosis and post-inflammatory regeneration, as reviewed previously. In reiteration, the dynamically-modulated key stages involved in wound healing are accomplished through a unique capability of the ECM to generate vast arrays of structures at the site of injury. The matrix therefore has a major influence on cellular processes, binds specific growth factors and cytokines and exquisitely organises the geometric framework, or scaffold, that can assist cell migration as well as numerous cell-to-cell interactions (Raghow, 1994; Hidalgo-Bastida and Cartmell, 2010).

4.3.1 SDS-PAGE Assay for Protein Separation

In addition to the studies that are focused on the cell development and proliferation in cultures, it was important to also consider the cytokines and growth factors that are involved in the healing process. The supposition is that there exists a status of dysregulation of acute-phase proteins within those people who smoke, contributing to the clinical problems of non- and delayed-union of healing bones. If the cytokines can be firstly identified in the fracture

haematomas, the quantitative and qualitative differences may reveal which mediators of healing are indeed affected by tobacco smoking. This will serve to provide a proteomic feature to the project, which is intended to fortify the cellular work that has been undertaken.

Glycine-SDS-PAGE (sodium dodecyl sulphate-polyacryamide gel electrophoresis), otherwise know as Laemmli SDS-PAGE, is often used to separate-out proteins of a molecular weight of up to 100 kiloDaltons (kDa), but is especially specific for the resolution of proteins smaller than 30 kDa. The electrophoretic system utilised in the resolution of proteins does so via the migration of the lysates through a polyacrylamide gel (16% acrylamide gels were used) under an electrical current (10-20mA), that was immersed in a tris/glycine, or Leammli, buffer. The captured proteins are then stained by using, for example, Coomassie blue or silver nitrate so that the resolved bands become clearly visible; they can then be photographed using a specialised gel imaging system (or digital camera) or stored (at 4˚C) for posterity in a solution of glycerol and distilled water for up to a couple of years (Laemmli, 1970; Schagger, 2006).

The determination of proteins from complex mixtures via electrophoresis is often undertaken as a preliminary action for proteomic analyses and the method represents an essential facet of modern research in many areas. Ideally, the technique employed would fractionate and isolate individual proteins on a more advanced, sensitive system known as 2D SDS-PAGE. The first dimension of the 2D system separates peptides via isoelectric focusing, whereby proteins migrate through a pH gradient. The second separation dimension is then applied using a

standard 1D process that resolves the proteins according to their molecular weight (Betgovargez *et al.*, 2005; Chuthapisith *et al.*, 2007).

In this investigation, a less complex version of the technique, 1D electrophoresis, was used to separate the haematoma serum, which is reported to contain around 600 extracellular proteins (Marshall *et al.*, 2004). This method was used because of its relative availability and reproducibility and proteins were, in the main, resolved successfully from the patient samples. After staining with either Coomassie blue or silver stain, several unknown bands appeared on the gels from the haematomas. Further investigations using mass spectrometry (QTOF, lcms) revealed that these were haemoglobin alpha and beta chains (Hb α and Hb β) (~14 kDa) and haptoglobin (~20 kDa). These proteins were present in substantial amounts, most likely due to intravascular or extravascular haemolysis that was taking place within the fracture haematoma environment. Whilst the haemoglobin chains are probably of no real significance in this study, other than an interesting observational characterisation of the microenvironment, haptoglobin is known to up-regulated by the liver in states of acute inflammation and trauma and is, therefore, classified as an acute-phase protein within its own right (O'Connell *et al.*, 2005). However, haptoglobin does have a major role in the formation and breakdown of red blood cells and is a natural component part of this connective tissue. This widespread haemolysis of the haematoma, as seen in all collected samples, is likely to be the reason why fracture haematomas are present at the fracture site for a few days only. The fracture haematoma is present at the milieu of repaired fractures for 3-5 days only; it is quickly broken down by enzymes at the site of injury which in turn causes this localised haemolysis (Quaye, 2008).

The tactic of purposely overloading the gels was undertaken, as it was thought that resolving more serum and therefore more acute-phase proteins, would result in an increased amount of the cytokines of interest apparent within the gel, but this did not appear to be the case. Western blotting was performed to probe individual analytes for TGF-β and IL-6 on SDS-PAGE gels, but both assays failed to transfer these proteins to a detectable level, despite a number of attempts (details not reported) and remained illusive on the nitrocellulose acetate membrane. Further studies that would identify the acute phase proteins expressed in trauma would be therefore advantageous. More specific techniques, for example 2D gel electrophoresis or liquid chromatography, would be required to gain a heightened level of resolution that is perhaps lacking in the 1D system for this particular study.

4.3.2 Intracellular Growth Factors

Growth factors are vital for regulating a variety of cellular processes and act as cell signalling molecules (see *Introduction*). For the circulatory system and bone marrow, in which cells are free and unbound, it is preferable for them to communicate by soluble, circulating protein molecules (Cross and Mustoe, 2003). In this study, intracellular TGF-β and IL-6 were detected in the cytoplasm of MSCs using flow cytometry. The protocol was semi-quantitative, so could not offer any definitive numerical differential conclusion as to the amounts of these proteins that could be compared between smokers and non-smokers. Only those cells positive for the expression of the proteins could be determined; i.e. if there was *any* amount of these proteins present in the cells, which was almost all of the

cells in all samples. A flow cytometer is capable of intracellular protein quantitation, but requires a dedicated protocol, a specialised commercially-available kit and greater expertise. The ELISA technique was instead selected to quantify specific proteins of interest due to its simplicity, affordability and widespread availability.

4.3.3 ELISA: VEGF and IL-6

Vascular endothelial growth factor gives rise to angiogenesis, contributing to a crucial occurrence in fracture repair, albeit that the mitogenic action of VEGF is largely restricted to vascular endothelial cells. It also increases endothelial cell permeability, leading to (neo)extravasation of fibrinogen and subsequent formation of a fibrin matrix, inducing MSC migration. This, coupled with endothelial cell proliferation, constitutes a potent angiogenic response that is seen in fracture repair. The chemotactic function of interleukin-6 on inflammatory mediators, in addition to its influence on the recruitment of MSCs in the early phase of trauma, is crucial for the initiation of the acute-phase response. Importantly, IL-6 peaks at 24 hours post-fracture, but is barely detectable after three days, which was a major factor in collecting the samples from the trauma patients within 24 hours of injury in the current study.

The preliminary data obtained from the ELISAs showed a 10% reduction in the amount of VEGF-A in smokers' serum, which was compounded with a lowered expression in the quantity of cytoplasmic IL-6, by approximately 15%. If patients are deficient of these growth factors, as was found by using ELISA, then this

could contribute to the delayed union that is synonymous with tobacco smoking and the healing of bones. The concept that the ECM of smokers' fracture sites is deficient of VEGF and is of inferior quality and consequently less able to deliver adequate angiogenesis for tissue repair, is feasible in light of these results where serum was involved. Given that a main source of VEGF is derived from residual bone marrow MSCs (Kagiwada *et al.*, 2008), which were found to proliferate at a slower rate in smokers' cells, it may be assumed that certain growth factors are not actually expressed as abundantly by MSCs and are impeded due to the compounded, tobacco-induced, toxic processes impacting upon them. In addition, cytoplasmic IL-6 down-regulation in the smoke-affected MSCs likely prevents chemotactic recruitment of further MSCs and mediators to the microenvironment, relaying an additional insult to the reparatory systems that are *in situ*, since IL-6 is known to initiate the healing process. The preliminary findings that the cells and molecules observed in this study are inferior in terms of quantity and quality from smokers suggests that other mediators may also be altered in a detrimental way. Further investigations are now justified to comprehensively study those additional factors that are affected by tobacco smoking and the fracture healing process. The ELISA data is considered preliminary due to a low sample size, so should be repeated using more samples.

4.1 Biochemical Analysis of the Fracture Haematoma Serum

The biochemical assays that were carried out in this study have concentrated on measuring the levels of b-ALP, albumin and total protein in serum that was derived from the fracture haematoma of smoking (n=10) and non-smoking (n=10)

patients. The assays were therefore designed to make direct comparisons between smokers and non-smokers. The results of the assays showed that b-ALP was 20% higher in smokers' fracture serum, although this was not statistically significant, probably because of high levels of variance within the groups. There were, however, significantly reduced levels of albumin ($p<0.05$) and highly significant lower amounts of total protein (albumin + globulin) ($p<0.01$) in smokers' serum in comparison to non-smokers'.

4.1.1 Bone-Specific Alkaline Phosphatase (b-ALP)

Bone-specific alkaline phosphatase is synthesized by osteoblasts and levels in serum can be raised in certain bone disease states. This is largely due to increased osteoblastic activity and is for this reason seen in bone metastases and hyperparathyroidism. Raised b-ALP levels were found in smokers' fracture serum, which perhaps alludes to increased osteoblastic activity in patients who smoke. This demonstrates an up-regulation of bone remodelling, which is likely due to compensatory mechanisms taking place because of cell destruction, as was shown to be the case in CSE-treated MSC cultures in the necrosis study (Figure 55 (a-c), Ch.3). It could also account for the NO imbalances that were found, which provides the basis for abnormal bone remodelling.

The 20% increase in serum levels of b-ALP in smokers are in line with the results found by Frost-Peneda *et al*. (2010), who showed elevated levels of alkaline phosphatase in serum derived from the peripheral blood of smokers compared to non-smokers. Their study was conducted using a large sample size (smokers

(*n*=3685) vs. non-smokers (*n*=1081)) and was an important investigation into the biomarkers of potential harm (BOPH) among adult smokers and non-smokers. The actual circulatory ALP levels were found to be 10.8% higher than non-smokers and this was highly statistically significant ($p<0.0001$). However, it was stated that age, race, gender, body mass index (BMI) and number of cigarettes smoked per day all had an effect on ALP (Frost-Pineda *et al.*, 2010).

The work by Frost-Pineda *et al*. (2010) was concentrated on circulatory ALP, which is mainly synthesised by the liver in the human subjects. Our study quantitated levels from actual fracture sites, thus would mainly have consisted of localised b-ALP and therefore negligible levels of circulatory ALP, perhaps rendering this study more orthopaedically-relevant. The level of raised b-ALP was higher than that found by Frost-Pineda and co-workers (b-ALP 20.0% in the current study compared with ALP 10.8% shown by Frost-Pineda *et al*. (2010)), suggesting that the problem is more pronounced at the fracture site than it is at the peripheral level. Irrespective of this, it shows that patients who smoke have an abnormal level of bone remodelling when compared to non-smokers, reflective of compensatory mechanisms taking place because of damage at the cellular level.

4.1.2 Albumin and Total Protein

Albumin and total protein, the latter being the amount of albumin and globulin combined in serum, were the subject of quantitative assays on the serum extracted from the haematoma samples. Albumin is the main component of plasma proteins and has an essential role in the maintenance of osmotic pressure. Importantly, it is

a major transporter of a large number of products, including calcium, bilirubin and hormones. It is used as a prognostic indicator of the transported products in terms of deteriorating function and inflammation. Decreases in albumin levels are attributable to malnutrition and hepatic synthesis alteration. Serious losses can be caused by trauma, kidney disease and cancer. Total protein is useful for monitoring gross changes in protein levels caused by a range of disease states, with decreased levels indicative of terminal liver failure (Doumas and Peters, 1997).

In the current study, there were statistically-significant reduced levels of both serum albumin ($p<0.05$) and total protein ($p<0.01$) in the fracture serum of smokers compared with the non-smoking patient group. In contrast, mean albumin levels in the smokers in the study by Frost-Pineda *et al*. (2010) remained unchanged when compared to non-smoker levels. This is probably due to their study not being concerned with trauma patients who are in the *acute phase*, as is the case with our data. Albumin is negatively expressed in trauma and this was found to be exaggerated in smokers serum, giving less scope for the transportation of calcium and other essential materials, such as acute phase proteins, to the fracture site. This is likely to contribute further to delayed healing that is seen so often in those patients who have sustained a fracture that smoke.

4.2 Conclusions

Fracture repair is a complex process involving many factors and molecules that are present in situ and recruited to the site of injury during the inflammatory, proliferative and maturation phases of healing. The effects of smoking are thought to be mediated via the vasoconstrictive and platelet-activating and aggregating properties of nicotine, the hypoxia-promoting effects of CO and the inhibition of oxidative metabolism by HCN at the cellular level.

There is no doubt that serious complications exist in fracture healing due to smoking and cessation of the habit perioperatively can improve the prognosis for smokers. Whilst clinical studies have addressed this problem very well, little work on the biochemical aspects has been conducted. The cellular and molecular processes involved in fracture healing are thus undoubtedly compromised and this problem has been the focus of this report.

Though much of the work undertaken was similar to that of a pilot study, it was found that smokers, whether via the smoking model or by direct comparison, were found to be disadvantaged due to detrimental changes in cellular morphology in terms of fragmentation and thinning, in addition to reduced proliferation and increased necrosis. Cells subject to the *in vitro* smoking environment were shown to possess lower levels of nitric oxide; such deficiencies are known to contribute to abnormal bone remodelling and poor vascularisation and vasodilatation.

Cessation of smoking is known to have a beneficial influence in fractures and this was apparent in the cellular recovery assays. The infusion of exogenous growth factor, BMP-2, offered a level of improvement to cellular viability in a smoking environment, offering hope to patients who are otherwise blighted with fracture pathologies, such malunion, nonunion and delayed union.

Proteomic studies showed that the serum possessed numerous acute-phase proteins, but the study would benefit from 2D electrophoresis in order to further isolate proteins of interest; the quantitation of intracellular proteins in MSCs would also be an advantage in future studies.

In biochemical analysis, b-ALP was found to be higher in smokers, confirming an abnormally-raised rate of bone remodelling in those patients. Serum albumin was significantly lowered in the same patients, signifying a reduced capacity for calcium transport.

In summary, tobacco smoking is known to have a deleterious effect on the dynamics of both wound and fracture healing and numerous studies involving animals and humans have been undertaken to demonstrate this. Orthopaedic surgeons have recommended that there should be a period of smoking cessation prior to surgery and, therefore, a history of cigarette smoking should be obtained from individuals so that the risks and complications may be discussed in detail. Both the orthopaedic surgeon and patient should, therefore, be completely aware of the harmful effects of smoking when assessing or planning a surgical intervention.

5 FUTURE STUDIES

5.1 Future Studies

Future cellular analyses would concentrate on the proteins that are synthesised by them as cell messengers in the fracture milieu. The protein profile in the haematoma is due to two mechanisms, those produced by the liver and mobilised to the fracture site and those produced by cells and platelets at the site of injury. Since the haemolysis of the haematoma may prevent accurate study of proteins within it, cultured cells offer a viable alternative. The effects of smoking can be synthesised using CSE in order to introduce a causative factor in cell damage and cell proteins may be observed following exposure to the toxic reagent using a variety of techniques. To improve statistical significance, all assays in this research should be repeated using more fracture haematoma specimens so as to increase the sample size, so that each assay has a sample size of sufficient statistical power and also to minimise variance. Additional sites within the hospital trust should be approached to widen the donor pool. Ethical approval has been granted from the Local Research Ethics Committee (LREC) to do this (see Appendices) and additional chief investigators at these sites have agreed to participate. However, using commercially-available osteoblastic cell lines may offer a degree of flexibility.

In order that the acute-phase proteins may be studied in more detail, they may be analysed in the fracture haematoma serum using a 2D electrophoresing system for an enhanced resolution. Due to haemolysis in samples, peripheral blood could be procured from participants to see if there are any differences in the quantity of circulating acute-phase proteins in the trauma phase. Western blotting can isolate

specific proteins of interest in both the haematoma serum and cell-culture supernatant prior to analysis using other techniques such as MALDI-TOF and ELISA, which are very useful methods in the quantitation of cytokines that are involved in fracture repair. MALDI-TOF can quantitate up to 5000 proteins per assay, making it the gold standard for protein analysis. However, flow cytometric probing and analysis of intracellular proteins could also measure membrane and intracellar proteins, DNA and RNA.

Cotinine is an alkaloid found in tobacco and is also a metabolite of nicotine. It is used as a biomarker for exposure to tobacco. When conducting studies comparing smokers with non-smokers, cotinine testing can be undertaken in the urine of consenting patients to quantitate nicotine levels. This would negate the requirement of relying on patients' honesty regarding number of cigarettes smoked per day. This could give an insight into the differences in fracture repair that may exist between a heavy or light smoking habit. However, the drawback of this is that patients using nicotine replacement therapy could produce a false positive.

This said, revision and refinement of the existing protocols in the study should be considered employing more rigorous scientific methods. Undertaking the following laboratory procedures is suggested overleaf.

Using Coverslips in Immunohistochemical Assays

Culturing cells on glass coverslips within flasks instead of using a cyto-spin instrument enables greater detail to be studied. Cells can then be fluorescently stained to identify various organelles, for example:

- Lysosomes
- Plasma membrane
- Golgi apparatus
- Nucleus, especially DNA.
- Smooth Endoplastic Reticulum
- Rough Endoplastic Reticulum
- Mitochondria

Various commercial biochemical stains are available in order that organelles may be observed under fluorescent microscopy, for example Using 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to A-T rich regions in DNA.

Non-Expressing Cell Line

The acquisition of a non-expressing cell line, such as the CD34+ haematopoietic stem cell, could also be included in control studies to show that the MSC cell surface antigen markers use in this study (CD29, CD44, CD73, CD105 and CD166) bind exclusively to membrane antigens expressed only by cells extracted and cultured from the fracture haematomas. This would give further credibility that cells are of an osteoblastic nature.

Cell Cycle Analysis/Population Doubling Time

Analysis of a large variety of cells in culture populations has revealed considerable variability in the lengths of their cell cycles. It would therefore be advantageous to be able to study the cell cycle and the phase distribution (G_1, S, G_2, M) to see if there are any differences between smokers (or CSE treated) and non smokers (or non-treated) in terms of cycle length or if more cells were in the dormant phase (G_0) in cultures. Cell cycle analysis can be achieved via flow cytometry, which provides a rapid and non-radioactive method for looking at cell proliferation. Maximum passage potential could also be investigated by continuously subculturing cells until senescence/quiescence is observed.

Differentiation Potential

In order to satisfy the criteria set out by the ISCT, isolated and cultured MSCs should be differentiated into osteocytes (osteogenic induction), chondrocytes (chondrogenic induction) and adipocyte (adipogenic induction). Traditional *In vitro* culturing techniques outlined as follows:

- Osteogenic Induction: Media containing dexamethasone, glycerphosphate, ascorbic acid. Stain with alizarin red.
- Chondrogenic Induction: Chondrogenic medium (high glucose DMEM) with dexamethasone, ascorbic acid-2-phosphate, praline, recombinant TGF-β and BMP-6. Stain with toluidine blue.
- Adipogenic Induction: Adipogenic medium containing 3-isobutyl-1 methylxanthene, insulin and indometacin. Stain with oil red.

(Oe *et al.*, 2007)

Validation of CSE Production

Additional studies could involve further validation of the CSE via mass-spectrometry to obtain an elementary profile of the toxins within the solution; this would enable a degree of quality assurance for reproducibility. The use of an electric pump system is likely to improve on the accuracy provided by the syringe method employed in this study. Increasing the CSE exposure time to cells may give an insight to the chronic effects of smoking on MSCs.

CHAPTER 6

REFERENCES

6 REFERENCES

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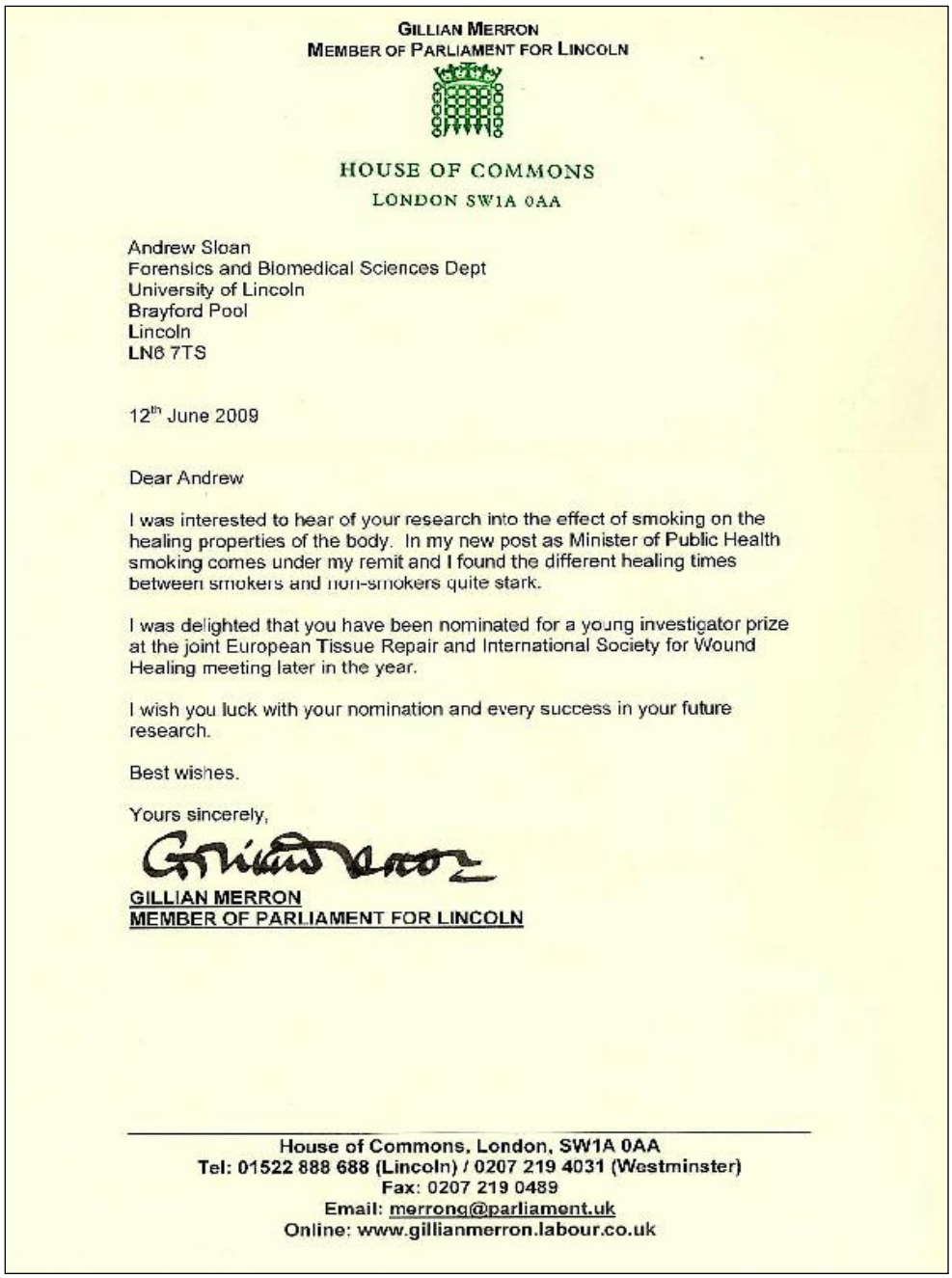
CHAPTER 7

APPENDICES

7 APPENDICES

7.1.1 Letter from Minister of Public Health



GILLIAN MERRON
MEMBER OF PARLIAMENT FOR LINCOLN

HOUSE OF COMMONS
LONDON SW1A 0AA

Andrew Sloan
Forensics and Biomedical Sciences Dept
University of Lincoln
Brayford Pool
Lincoln
LN6 7TS

12th June 2009

Dear Andrew

I was interested to hear of your research into the effect of smoking on the healing properties of the body. In my new post as Minister of Public Health smoking comes under my remit and I found the different healing times between smokers and non-smokers quite stark.

I was delighted that you have been nominated for a young investigator prize at the joint European Tissue Repair and International Society for Wound Healing meeting later in the year.

I wish you luck with your nomination and every success in your future research.

Best wishes.

Yours sincerely,

GILLIAN MERRON
MEMBER OF PARLIAMENT FOR LINCOLN

House of Commons, London, SW1A 0AA
Tel: 01522 888 688 (Lincoln) / 0207 219 4031 (Westminster)
Fax: 0207 219 0489
Email: merrong@parliament.uk
Online: www.gillianmerron.labour.co.uk

7.1.2 SICOT Conference Attendance

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Andrew SLOAN

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SICOT

CERTIFICATE OF ORAL PRESENTATION

This is to certify that **Andrew SLOAN** has presented an abstract entitled

THE BIOCHEMICAL EFFECTS OF TOBACCO SMOKE ON FRACTURE HEALING: AN IN VITRO MODEL

Authors: Andrew SLOAN, Issam HUSSAIN, Mohammad MAQSOOD, Oleg EREMIN and Mohamed EL-SHEEMY

at the SICOT 2011 XXV Triennial World Congress held at the Prague Congress Centre in Prague, Czech Republic, from 6 to 9 September 2011.

Prague, 9 September 2011

Prof Jochen Eulert
Secretary General

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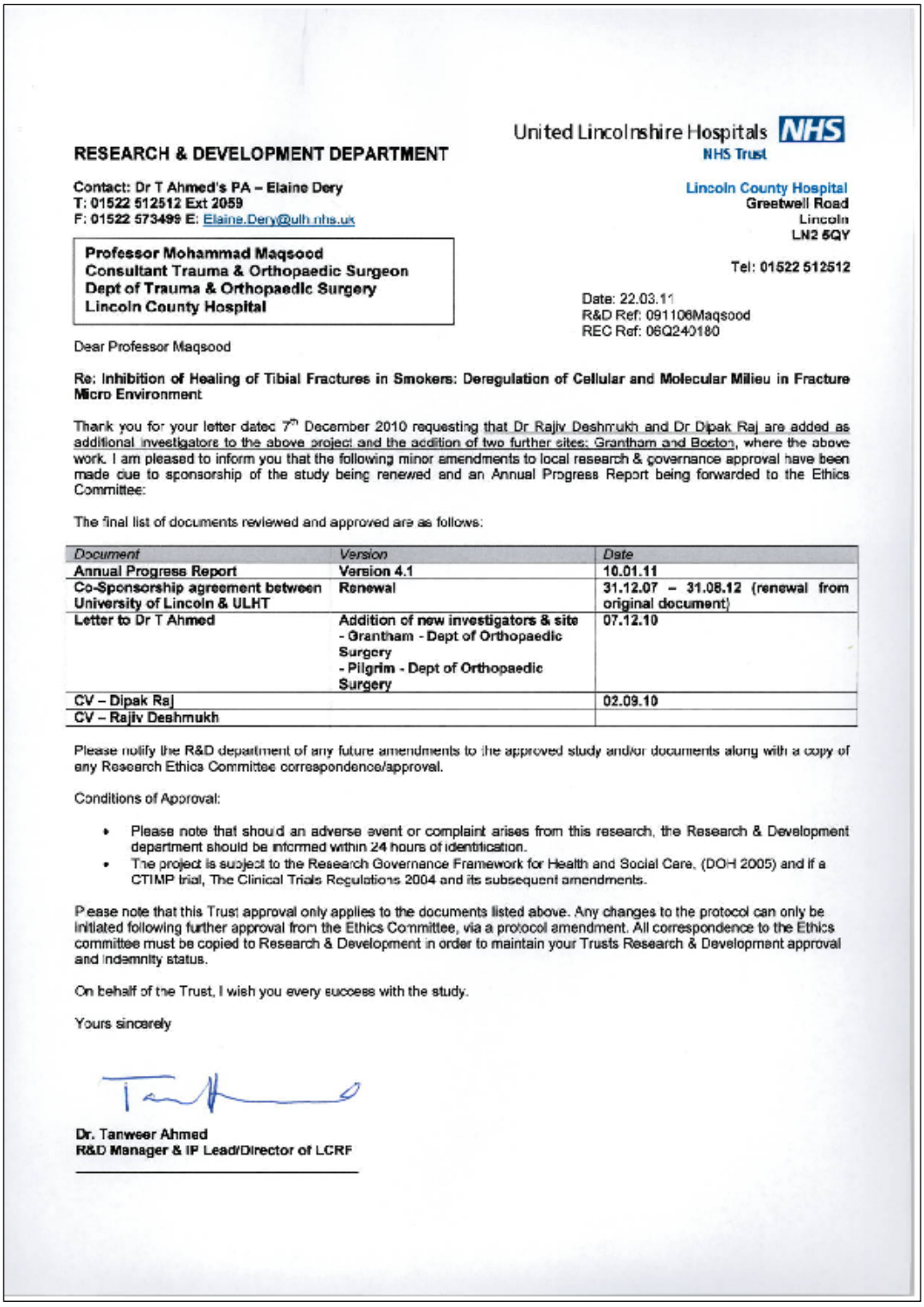
7.1.3 Ethical Approval Documentation



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RESEARCH & DEVELOPMENT DEPARTMENT

Contact: Dr T Ahmed's PA – Elaine Dery
T: 01522 512512 Ext 2059
F: 01522 573499 E: Elaine.Dery@ulh.nhs.uk

Professor Mohammad Maqsood
Consultant Trauma & Orthopaedic Surgeon
Dept of Trauma & Orthopaedic Surgery
Lincoln County Hospital

Lincoln County Hospital
Greetwell Road
Lincoln
LN2 5QY

Tel: 01522 512512

Date: 22.03.11
R&D Ref: 091106Maqsood
REC Ref: 06Q240180

Dear Professor Maqsood

Re: Inhibition of Healing of Tibial Fractures in Smokers: Deregulation of Cellular and Molecular Milieu in Fracture Micro Environment

Thank you for your letter dated 7th December 2010 requesting that Dr Rajiv Deshmukh and Dr Dipak Raj are added as additional investigators to the above project and the addition of two further sites: Grantham and Boston, where the above work. I am pleased to inform you that the following minor amendments to local research & governance approval have been made due to sponsorship of the study being renewed and an Annual Progress Report being forwarded to the Ethics Committee:

The final list of documents reviewed and approved are as follows:

Document	*Version*	*Date*
Annual Progress Report	**Version 4.1**	**10.01.11**
Co-Sponsorship agreement between University of Lincoln & ULHT	**Renewal**	**31.12.07 – 31.08.12 (renewal from original document)**
Letter to Dr T Ahmed	**Addition of new investigators & site - Grantham - Dept of Orthopaedic Surgery - Pilgrim - Dept of Orthopaedic Surgery**	**07.12.10**
CV – Dipak Raj		**02.09.10**
CV – Rajiv Deshmukh		

Please notify the R&D department of any future amendments to the approved study and/or documents along with a copy of any Research Ethics Committee correspondence/approval.

Conditions of Approval:

- Please note that should an adverse event or complaint arises from this research, the Research & Development department should be informed within 24 hours of identification.
- The project is subject to the Research Governance Framework for Health and Social Care, (DOH 2005) and if a CTIMP trial, The Clinical Trials Regulations 2004 and its subsequent amendments.

Please note that this Trust approval only applies to the documents listed above. Any changes to the protocol can only be initiated following further approval from the Ethics Committee, via a protocol amendment. All correspondence to the Ethics committee must be copied to Research & Development in order to maintain your Trusts Research & Development approval and indemnity status.

On behalf of the Trust, I wish you every success with the study.

Yours sincerely

Dr. Tanweer Ahmed
R&D Manager & IP Lead/Director of LCRF

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To: Andrew Sloan

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Andrew S... RE: Obtai... malunion... PhDthesis... EndNote 9

18:23 Friday 23/08/2013

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Reply Reply All Forward

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Safdar, Sheik - Hoboken [ssafdar@wiley.com]

To: Andrew Sloan

Friday, August 23, 2013 9:01 PM

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A01_First_Name: Andrew
A02_Last_Name: Sloan
A04_Address: 71 Walcott Road
A05_City: Lincoln
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A08_Country: UK
A09_Contact_Phone_Number: +441526861394
A10_Fax:
A11_Emails: asloan@lincoln.ac.uk

Andrew S... RE: Educa... PhDthesis... EndNote 9 HP v125w...

21:17 Friday 23/08/2013

EA2

Ethical Approval Form: Human Research Projects

Please word-process this form, handwritten applications will not be accepted

UNIVERSITY OF LINCOLN

This form must be completed for each piece of research activity whether conducted by academic staff, research staff, graduate students or undergraduates. The completed form must be approved by the designated authority within the Faculty.mailto:jgreen@lincoln.ac.uk

Please complete all sections. If a section is not applicable, write N/A.

1 Name of Applicant	ANDREW STEPHEN SLOAN	
	Department: NATURAL AND APPLIED SCIENCES	Faculty: FACULTY OF HEALTH, LIFE AND SOCIAL SCIENCES
2 Position in the University	Postgraduate Researcher	
3 Role in relation to this research	PhD Student	
4 Brief statement of main Research Question	The key question that is hoped to be answered is; what are the biological adverse effects of smoking on the microenvironment of healing bone fractures?	
5 Brief Description of Project	The research will concentrate on the inhibition of healing of tibial fractures in smokers. Smoking can delay the healing process of bone injuries and in some cases can account for the nonunion of a fracture. Extended healing times are a serious complication, resulting in adverse effects in quality of life and morbidity, leading to socio-economic problems.	
	Approximate Start Date: April 2007	Approximate End Date: July 2012
6 Name of Principal Investigator or Supervisor	Dr. Mohamed El-Sheemy	
	Email address: Melsheemy@lincoln.ac.uk	Telephone: Tel 01522 5733373 (dir) Fax 01522 573499
7 Names of other researchers or student investigators involved	**2.** Professor O Eremin **3.** Professor M Maqsood	

8 Location(s) at which project is to be carried out	1. Biomedical Research Laboratory, University of Lincoln 2. Lincoln County Hospital, Greetwell Road, Lincoln 3. Boston Pilgrim Hospital, Sibsey Road, Boston 4. Grantham & District Hospital, Manthorpe Road, Grantham

9 Statement of the ethical issues involved and how they are to be addressed –including a risk assessment of the project based on the vulnerability of participants, the extent to which it is likely to be harmful and whether there will be significant discomfort. **(This will normally cover such issues as whether the risks/adverse effects associated with the project have been dealt with and whether the benefits of research outweigh the risks)**	The research will concentrate on the inhibition of healing of tibial fractures in smokers. Smoking can delay the healing process of bone injuries and in some cases can account for the nonunion of fractures. Extended healing time is a serious complication, resulting in adverse effects in quality of life and morbidity, leading to socio-economic problems. In order to gain supporting data to confirm our hypothesis that smoking does cause delayed healing or nonunion of bone, a series of laboratory tests will be undertaken. These may include; Specialised cell culturing Antibody testing techniques Protein analyses Assessment of in vitro growth factor inclusion in cell cultures (BMP-7, TGF-beta, VEGF) If we can understand the adverse effects at the molecular and cellular at the fracture site, then this can help the patient overcome the healing complications associated with smoking. This study hopes to provide an understanding of the deregulation and dysfunction of key cells and regulatory molecules at the time of traumatic haematoma formation in those patients who smoke. The research addresses an important clinical dilemma, which has been poorly studied at the biological level. Recent data show significant reduction rates in healing of bone fractures in smokers, which has been evident in both long bones and the bones of the vertebral column. A previous study found a 69% delay in radio-graphic union of tibial fractures

	in smokers compared with non-smokers. Other research has shown a 60% increase in healing time to achieve stable clinical union following cast immobilisation of tibial fractures. In smokers, there is a reduced bone mass density (BMD) throughout the skeleton, particularly affecting the hip. Meta-analysis has demonstrated that those actively smoking are at increased risk of fractures Those patients taking anticoagulants, steroids or anti-inflammatory drugs in the three months prior to injury will be excluded from the trial. The study will involve adult patients from each group (i.e., smokers and non-smokers). The age of each patient, ethnic group and daily number of cigarettes smoked (where applicable) is to be noted and logged and will accompany each sample. Informed consent from all patients, as well as ethical clearance from the NHS Research Ethics Committee has been granted. Main Ethical Risk: After exposure of the fracture site, the haematoma will be carefully removed manually and placed in a sterile polypropylene container by competent theatre staff at the Fracture Clinic, Lincoln County Hospital. There will be no increased exposure to microbial pathogens or detrimental effect on the healing process as a result of sample excision. Researchers are immunised against Hepatitis B.

Ethical Approval From Other Bodies

10 Does this research require the approval of an external body ?	**Yes** **If "Yes", please state which body:-NHS RESEARCH ETHICS COMMITTEE**
11 Has ethical approval already been obtained from that body ?	**YES Yes -Please append documentary evidence to this form** **If "No", please state why not:-** **Please note that any such approvals must be obtained and documented before the project begins.**

APPLICANT SIGNATURE

I hereby request ethical approval for the research as described above.
I certify that I have read the University's ETHICAL PRINCIPLES FOR CONDUCTING RESEARCH WITH HUMANS AND OTHER ANIMALS.

16 SEP 2010

_____
Applicant Signature

ANDREW STEPHEN SLOAN

_____
PRINT NAME

FOR COMPLETION BY THE CHAIR OF THE FACULTY RESEARCH COMMITTEE

Please select ONE of A, B, C or D below:

☐ **A. The Faculty Research Committee gives ethical approval to this research.**

☐ **B. The Faculty Research Committee gives conditional ethical approval to this research.**

12 Please state the condition (inc. date by which condition must be satisfied if applicable)	

☐ **C. The Faculty Research Committee can not give ethical approval to this research but refers the application to the University Research Ethics Committee for higher level consideration.**

13 Please state the reason	

☐ **D. The Faculty Research Committee can not give ethical approval to this research and recommends that the research should not proceed.**

7.1.5 Patient Information Sheet and Consent Form

UNIVERSITY OF LINCOLN

United Lincolnshire Hospitals NHS
NHS Trust

Research Project: The Inhibition of Fracture Healing in Smokers

Tobacco smoking has been shown to have a detrimental impact on fracture healing and is often associated with poor bone repair. Whilst a lot of research has concentrated on the clinical aspects of fracture healing and smoking, very few studies have been undertaken on human tissue. The aims of this research project are to assess the impact of tobacco smoking on the biological aspects of bone healing. This will be done by analysing tiny amounts of donated human blood from the site of the fracture (called a 'haematoma') using a variety of laboratory techniques at The University of Lincoln. The information gained from the study is helping surgeons, scientists and patients to understand the impacts of smoking and how it affects the healing process.

We are asking patients who have sustained a fractured tibia to take part in this study by consenting to donate a small amount of haematoma from the fracture site. Your orthopaedic surgeon will collect this from you whilst you are undergoing your operation to reset your tibia, after you have been anaesthetised. This tissue is normally washed away during the operation, so your healing process will not be affected by its collection.

The collected fracture haematoma will then be studied in a scientific research laboratory at The University of Lincoln and your personal details will not be known to anyone other than your orthopaedic surgeon. You will not be contacted in the future regarding the study and you will remain anonymous throughout the research. You are free to withdraw from the project at any time if you wish to do so.

Many thanks indeed for your cooperation in this vital research.

United Lincolnshire Hospitals NHS
NHS Trust

UNIVERSITY OF LINCOLN

Research Project: The Inhibition of Fracture Healing in Smokers

CONSENT FORM *Please initial box*

1. I can confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without having to give reason and without my medical care and legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission to for these individuals to have access to my records. ☐

4. I agree to my General Practitioner (GP) being informed of my participation in this study. ☐

5. I agree to take part in this study. ☐

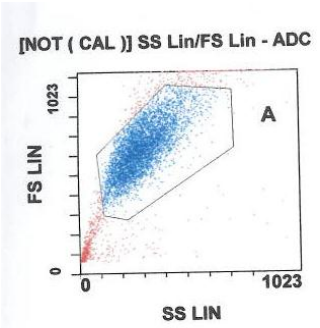
Name of Patient: Date: Signature:

Name of Person taking consent: Date: Signature:

Name of Researcher: Date: Signature:

When completed 1 for patient, 1 for researcher site file, 1(original) to be kept in medical notes.

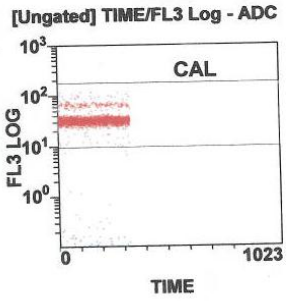
7.1.6 Flow Cytometry Data



[NOT (CAL)] SS Lin/FS Lin

Region	Cells/µL	Number	%Gated
A	1192	4882	82.66

Cells in Counts per ul

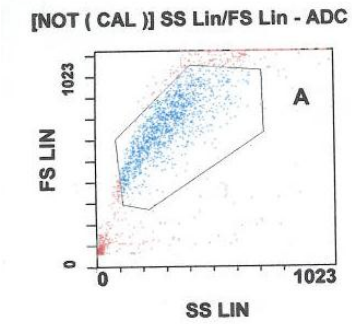


[Ungated] TIME/FL3 Log

Region	Cells/µL	Number	%Gated
CAL	1000	4094	40.94

[Ungated] Legend
29-APR-08
00001442 031.LMD

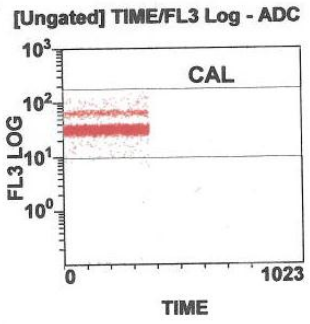
fibro like Cell CountPRO



[NOT (CAL)] SS Lin/FS Lin

Region	Cells/µL	Number	%Gated
A	159	1262	61.11

Cells in Counts per ul



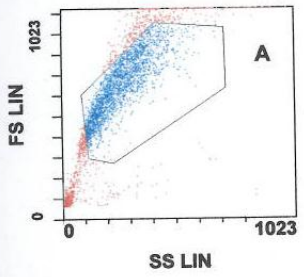
[Ungated] TIME/FL3 Log

Region	Cells/µL	Number	%Gated
CAL	1000	7935	79.35

[Ungated] Legend
04-APR-08
013a1stpass040408 00001416 014.LMI

fibro like Cell CountPRO

[NOT (CAL)] SS Lin/FS Lin - ADC

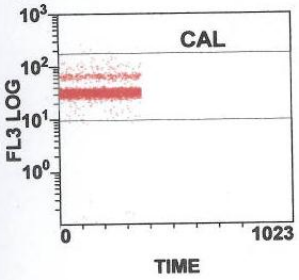


[NOT (CAL)] SS Lin/FS Lin

Region	Cells/µL	Number	%Gated
A	322	2158	65.26

Cells in Counts per ul

[Ungated] TIME/FL3 Log - ADC



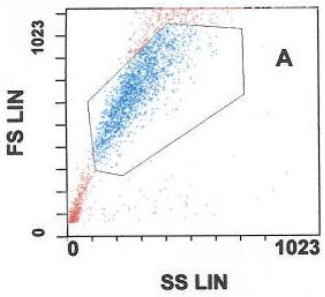
[Ungated] TIME/FL3 Log

Region	Cells/µL	Number	%Gated
CAL	1000	6693	66.93

[Ungated] Legend
04-APR-08
013b1stpass040408 00001417 015.LMI

fibro like Cell CountPRO

[NOT (CAL)] SS Lin/FS Lin - ADC

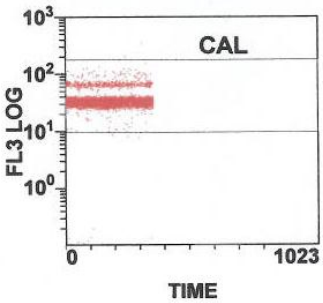


[NOT (CAL)] SS Lin/FS Lin

Region	Cells/µL	Number	%Gated
A	231	1691	62.98

Cells in Counts per ul

[Ungated] TIME/FL3 Log - ADC

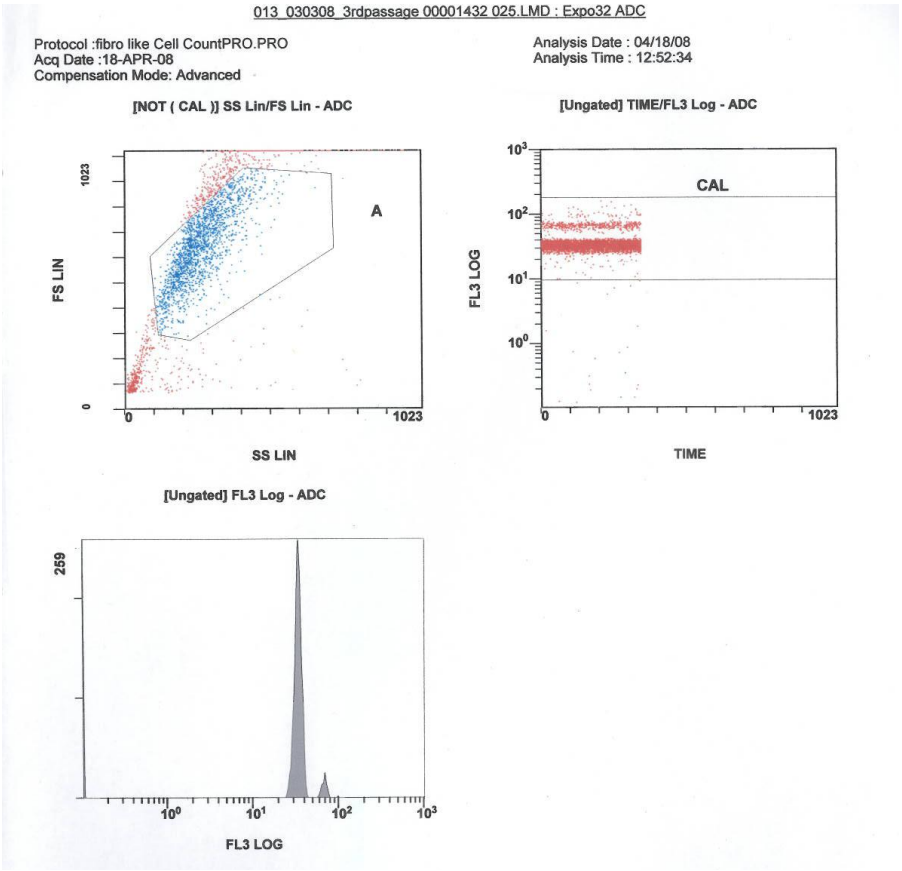


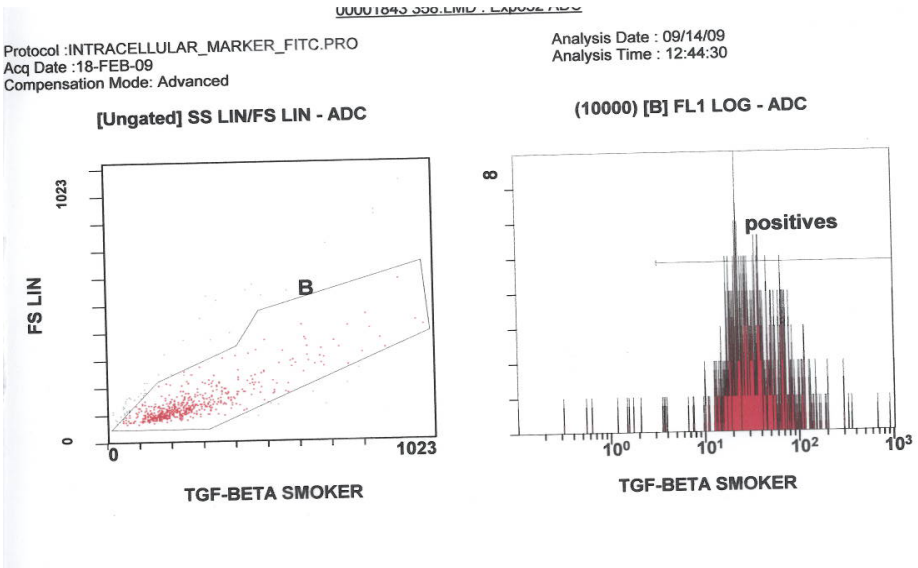
[Ungated] TIME/FL3 Log

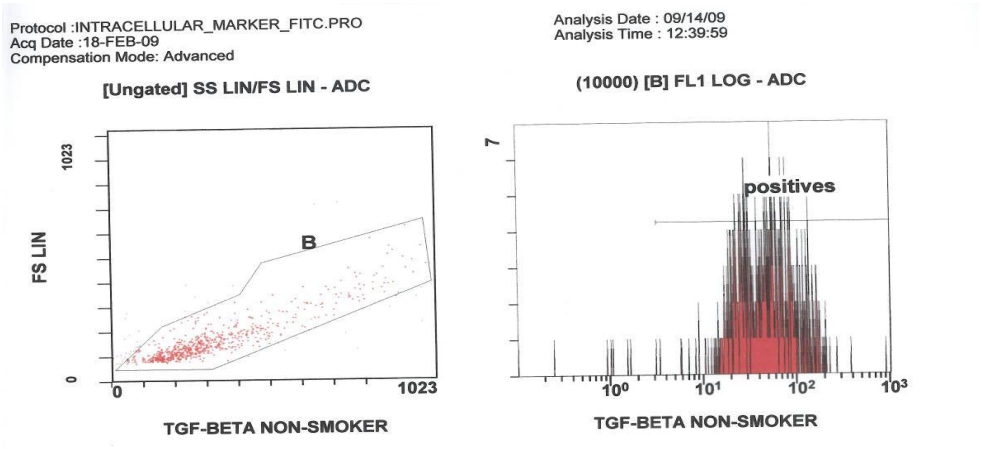
Region	Cells/µL	Number	%Gated
CAL	1000	7315	73.15

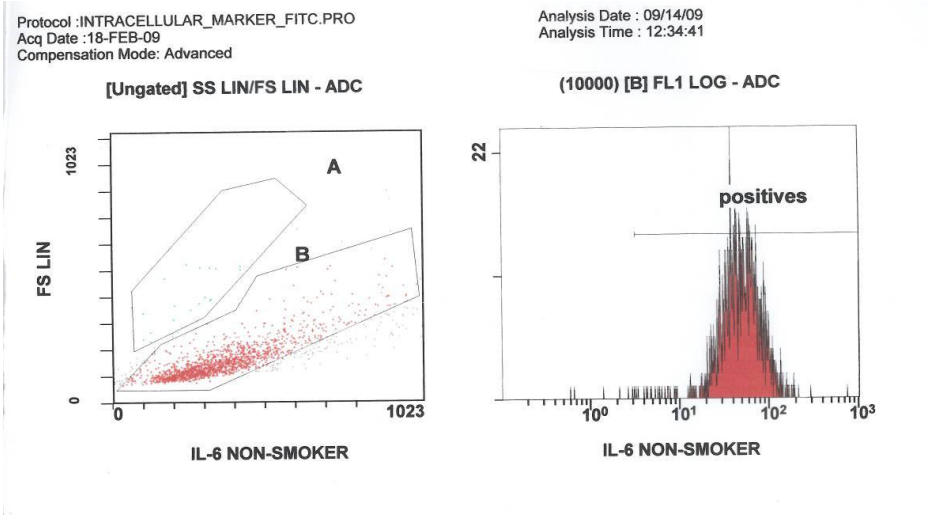
[Ungated] Legend
18-APR-08
013_030308_3rdpassage 00001431 02

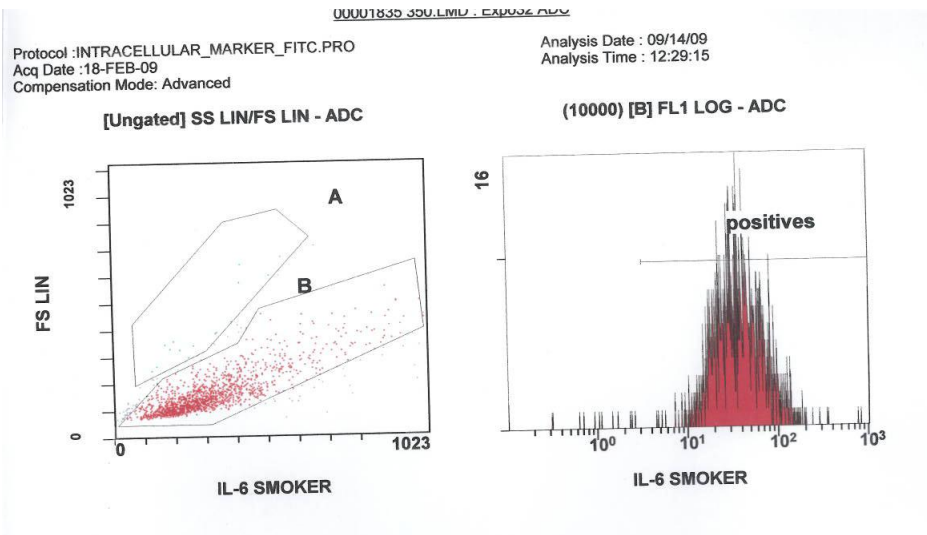
fibro like Cell CountPRO


013_030308_3rdpassage 00001432 025.LMD : Expo32 ADC
Protocol :fibro like Cell CountPRO.PRO
Acq Date :18-APR-08
Compensation Mode: Advanced
Analysis Date : 04/18/08
Analysis Time : 12:52:34
[NOT (CAL)] SS Lin/FS Lin - ADC
A
FS LIN
SS LIN
[Ungated] TIME/FL3 Log - ADC
CAL
FL3 LOG
TIME
[Ungated] FL3 Log - ADC
FL3 LOG

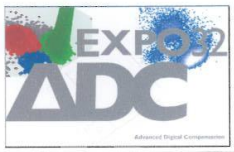

Protocol :INTRACELLULAR_MARKER_FITC.PRO
Acq Date :18-FEB-09
Compensation Mode: Advanced
Analysis Date : 09/14/09
Analysis Time : 12:44:30
[Ungated] SS LIN/FS LIN - ADC
B
FS LIN
TGF-BETA SMOKER
(10000) [B] FL1 LOG - ADC
positives
TGF-BETA SMOKER


Protocol :INTRACELLULAR_MARKER_FITC.PRO
Acq Date :18-FEB-09
Compensation Mode: Advanced
[Ungated] SS LIN/FS LIN - ADC
FS LIN
1023
0
B
0
1023
TGF-BETA NON-SMOKER
Analysis Date : 09/14/09
Analysis Time : 12:39:59
(10000) [B] FL1 LOG - ADC
7
positives
10⁰
10¹
10²
10³
TGF-BETA NON-SMOKER


Protocol :INTRACELLULAR_MARKER_FITC.PRO
Acq Date :18-FEB-09
Compensation Mode: Advanced
[Ungated] SS LIN/FS LIN - ADC
FS LIN
1023
0
A
B
0
1023
IL-6 NON-SMOKER
Analysis Date : 09/14/09
Analysis Time : 12:34:41
(10000) [B] FL1 LOG - ADC
22
positives
10⁰
10¹
10²
10³
IL-6 NON-SMOKER

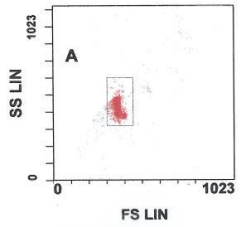

Protocol :INTRACELLULAR_MARKER_FITC.PRO
Acq Date :18-FEB-09
Compensation Mode: Advanced
[Ungated] SS LIN/FS LIN - ADC
FS LIN
1023
0
A
B
0
1023
IL-6 SMOKER
Analysis Date : 09/14/09
Analysis Time : 12:29:15
(10000) [B] FL1 LOG - ADC
16
positives
10⁰
10¹
10²
10³
IL-6 SMOKER

Enter Institution Name
Street Address
City, State, Zip



Flow Check Fluorospheres

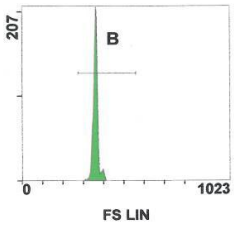
[Ungated] FS LIN/SS LIN - ADC



[Ungated] FS LIN/SS LIN

Region	Number	%Gated	X-Mean	HP X-CV
A	5000	92.08	361.2	1.8

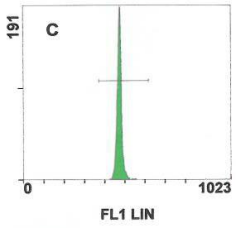
(5000) [A] FS LIN - ADC



(5000) [A] FS LIN

Region	Number	%Gated	X-Mean	HP X-CV
B	5000	100.00	361.2	1.8

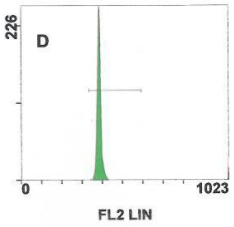
[A] FL1 LIN - ADC



[A] FL1 LIN

Region	Number	%Gated	X-Mean	HP X-CV
C	4824	96.48	474.6	1.5

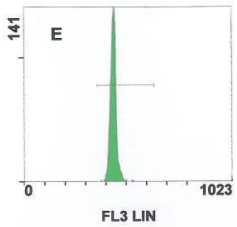
[A] FL2 LIN - ADC



[A] FL2 LIN

Region	Number	%Gated	X-Mean	HP X-CV
D	4771	95.42	387.4	1.5

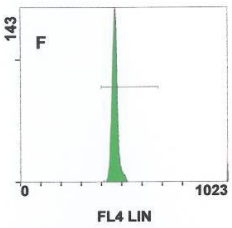
[A] FL3 LIN - ADC



[A] FL3 LIN

Region	Number	%Gated	X-Mean	HP X-CV
E	4866	97.32	440.0	2.7

[A] FL4 LIN - ADC



[A] FL4 LIN

Region	Number	%Gated	X-Mean	HP X-CV
F	4792	95.84	469.9	2.3